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UNIVERSITY OF ALBERTA
MAY - 3 1978
FACULTY OF GRADUATE STUDIES AND RESEARCH

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TITLE OF THESIS/*TITRE DE LA THÈSE* Investigation of the electrolytic method of radio-iodination of autologous human fibrinogen

UNIVERSITY/*UNIVERSITÉ* University of Alberta

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* Ph.D.

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE GRADE* 1978

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THE UNIVERSITY OF ALBERTA

INVESTIGATION OF THE ELECTROLYTIC METHOD OF RADIOIODINATION OF AUTOLOGOUS FIBRINOGEN

BY

© ANDREW MARK STEVENS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

BIONUCLEONICS

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

SPRING 1978

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled INVESTIGATION OF THE ELECTROLYTIC METHOD OF RADIOIODINATION OF AUTOLOGOUS FIBRINOGEN submitted by ANDREW MARK STEVENS in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in BIONUCLEONICS.

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TO MY MOTHER

ABSTRACT

Radioiodinated human fibrinogen has been used since 1964 to detect deep venous thrombosis in the legs of post-operative patients. However, the potential hazard of transmitting serum hepatitis with pooled fibrinogen has prevented the widespread use of radioiodinated fibrinogen in North America.

A method has been developed for isolating fibrinogen from a small sample of blood in an efficient and rapid procedure requiring two hours to complete. Several methods of isolating fibrinogen were developed and abandoned because they were found to be too time consuming.

The parameters influencing the yield of iodinated fibrinogen by the electrolytic method of radioiodination were examined. Approximately 80% of iodine could be incorporated into fibrinogen in 15 minutes, using an iodide to protein ratio of 1:2 at pH 7.0. The current, cell potential, anolyte volume, iodide and protein concentrations, and pH were found to be critical parameters for the reaction.

The labelled fibrinogen was tested for adverse effects of the labelling method by several in vitro techniques. It was found that there was an insignificant decrease in clottability and thrombin clotting time; electrophoresis showed no evidence of molecular damage. The hydrolysis rate of iodofibrinogen ranged between 2 to 4% per day at 37°C. The increase in molecular aggregates was minimal.

The fractional catabolic rates of labelled fibrinogen were found to be 26.2% per day in dogs, and 60.0% per day in rabbits. This was 42.5% lower than that found for three commercial preparations measured in rabbits.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to my supervisors, Dr. A. A. Noujaim and Dr. A. Shysh, for their patience and guidance during the course of this study.

Special thanks are extended to Mr. C. Ediss, who designed and built the electrolytic cells and associated electronics which were used in this thesis, and who wrote the computer programs for the analysis of the data. His numerous suggestions and explanations of things physical, mathematical and philosophical have been most invaluable to the successful completion of the present investigation.

Special thanks are also extended to Dr. U. K. Terner for his invaluable assistance with the animal work, to Dr. L. I. Wiebe for the use of the Pharmacia UV Flow Monitor and Chart Recorder, and to all those people who donated blood for this present work.

Financial assistance from Geigy (Canada) Limited in the form of a Geigy Pharmacy Scholarship, from Pfizer Pharmaceutical Division in the form of the Pfizer Research Scholarship, and from the University of Alberta in the form of a Teaching Assistantship are all gratefully Acknowledged.

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INTRODUCTION

It has been estimated that there are 630,000 cases of pulmonary embolism annually in the United States.(1) Prevention of embolism depends on early detection of deep venous thrombosis, and thus a considerable effort has been directed towards finding a simple and objective method for this purpose. While other physical and radio-isotopic methods of diagnosis of deep venous thrombosis are available, none have achieved the wide spread acceptance of the Fibrinogen Uptake Test.

It was observed by McFarlane (2) that fibrinogen could be isolated from plasma and labelled with I-131 without changing its metabolism *in vivo*. It was further observed by Hobbs and Davies(3) in 1960 that I-131 labelled fibrinogen could be detected in forming thrombi. This idea was adapted to the clinical situation by Palko *et al.*(4) who used I-131 labelled fibrinogen to confirm the presence of clinically suspected deep venous thrombosis in patients. Atkins and Hawkins(5) in 1965 labelled human fibrinogen with I-125 rather than I-131, because of its longer physical half life, a lower total body radiation dose delivered to the patients, and soft gamma radiation which allowed the use of light-weight portable detectors.

Several British surgeons including Flanc *et al.*(6), Negus *et al.*(7), and Kakkar *et al.*(8), labelled fibrinogen with I-125 and investigated the uptake of I-125 into post-operative deep venous thrombi and developed the protocol for the Fibrinogen Uptake Test. Other investigators soon began to use the technique for measurement of the incidence of deep venous thrombosis in clinical trials of various methods and drugs for the prophylaxis of deep venous thrombosis.

Fibrinogen is present in the blood of all vertebrates. It is the central protein in the coagulation system in which the soluble plasma protein is converted by thrombin into an insoluble polymerized fibrin gel. In humans, it is present at a concentration of 200-600(9) mg per 100 ml, and consists of a dimer with three polypeptide chains in each half(10). The molecular weight is commonly accepted to be 340,000, but the size and shape are a subject of lively debate(10). The data are consistent with a nodular structure with an axial ratio of 5-10, a length of 350-500 Å and a width of 50-80 Å (10).

During the isolation of fibrinogen from plasma, the most damaging contaminants are plasminogen and thrombin(11). The conversion of plasminogen to plasmin results in the degradation of fibrinogen, while the formation of thrombin from prothrombin results in the polymerization of fibrin. The processes of isolating fibrinogen from plasma have been lengthy and intricate, requiring either an extensive extraction procedure, such as the method of Bomback et al.(12) which uses glycine and alcohol fractionation to produce a number of fibrinogen fractions from Cohn Fraction I, or they require an initial time consuming period of adsorption of the prothrombin complex on several inorganic adsorbents such as the glycine precipitation method of Kazal et al.(13).

Fibrinogen is currently isolated on a commercial scale by the cold ethanol method of Cohn et al.(14). However, it has been found that this method also fractionates the Australia (hepatitis-associated) antigen with the fibrinogen.(15) Thus, if fibrinogen from a commercial source is used for the detection of deep venous thrombosis, there is a risk of transmitting serum hepatitis.

It has been reported by Krohn et al.(16,19), Metzger et al. (17,131), and Coleman et al.(18) that the method of radioiodination of fibrinogen profoundly affects the in vivo and in vitro properties of the labelled protein. The labelling method may produce a product with low clottability (16), a high rate of dehalogenation(16), oxidation of some amino acids(19), and contain a large portion of aggregated protein(16,19). When injected into laboratory animals, poor preparations will be rapidly cleared from the circulation and will accumulate in the liver and spleen(131). At the same time, the non-protein radioactivity will show a rapid increase(131).

The main methods of radioiodination of proteins or peptides containing tyrosine are the iodine monochloride method of McFarlane (20), the Chloramine T method of Hunter and Greenwood(21), the lactoperoxidase method of Marchalonis(22), and the electrolytic method of Rosa et al.(23). The latter method was introduced in 1964, and was described as having several advantages, including the absence of oxidizing agents and the ability to control the rate of iodine liberation.

The objective of this research was to isolate fibrinogen from a small sample of blood in such a way that a highly purified, sterile and pyrogen-free product was obtained. The protein was then to be radioiodinated without significant denaturation. The resulting drug should then have in vitro properties comparable with native fibrinogen, and should have a long clearance rate from the blood and should be stable in vivo. In this thesis, several methods of isolating human and canine fibrinogen were investigated. The parameters involved in the electrolytic method of radioiodination

of human fibrinogen were examined in detail. The *in vitro* properties of fibrinogen iodinated by this method were then characterized. Next, the electrolytically labelled protein was injected into dogs and the fractional catabolic rates were determined. The fractional catabolic rates of fibrinogen labelled by the electrolytic, iodine monochloride, and Chloramine T methods were determined in rabbits. Finally, some qualitative studies on the detection of surgically induced thrombi by the use of electrolytically radioiodinated fibrinogen were performed in dogs.

LITERATURE SURVEY

I. IODINE AND ITS CHEMISTRY

A. Iodine

Iodine was discovered in 1811 by Bernard Courtis and named "iode" by Gay-Lussac from the Greek meaning violet-coloured(24). Although it is classified as a rare element, it is the 47th most abundant element of the earth's crust (0.001%)(24). Natural iodine is entirely monoisotopic, occurring only as I-127. It is found in rocks, soils, and seawater, but is produced from seaweed, underground brines and Chilean nitrate deposits(24).

Electronic Structure:

Iodine has an atomic number of 53 and is the 4th halogen in the 7th group of the periodic table(26). The neutral atom has the electronic configuration of a krypton core plus 4d10 5s2 5p5(26). The s2p5 configuration of the outer shell is missing one electron of the noble gas configuration of s2p6. Thus, the atom has a strong tendency to attract a single electron to its outer shell, and only five elements are more electronegative(24).

Oxidation States:

While usually monovalent, the known oxidation states are -1 (I^- iodide); 0 (I_2 iodine); +1 (HOI hypoiodous acid); +4 (IO_2 iodine dioxide); +5 (HIO_3 iodic acid, IO_3^- iodate); +7 (HIO_4 periodic acid)(26).

Physical Properties:

Iodine is a greyish black solid with a metallic luster. At atmospheric pressure, it sublimes to form a violet vapor(26). It is soluble in water to the extent of 0.33 g/l (2.59×10^{-3} M/l) at 25°C,

and is highly soluble in organic solvents and aqueous KI solutions(26). Iodine dissolved in benzene is red, violet in CCl_4, $CHCl_3$, hexane, or CS_2, brown in aqueous KI and in ethyl ether, and blue in oleum and IF_5(24). It melts at 113.5°C to a black liquid and boils at 184°C (24). Since the atomic radius decreases in the order I>Br>Cl>F, it forms the following interhalogens, in which iodine is the central atom: IBr, ICl, ICl_3, IF_5 and IF_7(24).

B. Metabolism of Iodine

The only known physiological function of iodine is to form part of the thyroid hormones, triiodothyronine (T-3) and thyroxine (T-4) (28). Dietary iodine ultimately originates in the soil from which food and water are taken. An average daily intake of 150 ug is adequate to prevent goiter(28).

The human body contains 20 to 50 mg of iodine of which 25% is located in the thyroid(28). In the euthyroid adult, the turnover rate is 60-120 ug/day(27).

Iodine is present in the diet as iodine, inorganic iodide, and organically bound iodine. All of the inorganic iodide is absorbed from the lumen of the intestine; T-3 and T-4 are absorbed unaltered, but iodide in other organic compounds is thought to be converted to free iodide by the mucosa(27). Once in the plasma, it circulates as free iodide, where it is absorbed by the thyroid, salivary and gastric glands, mammaries, placenta and ovaries(27).

Iodide is abstracted from the plasma by the thyroid against a concentration gradient of 25:1 by the iodide pump(28). It is oxidized in the follicular cells and incorporated into the thyro-

globulin before secretion into the follicular colloid. The thyroidal hormones are released from thyroglobulin by lysosomal enzyme hydrolysis. Hormonal iodine is deiodinated during the metabolism of thyroid hormones or it passes through the liver with the hormones as they are conjugated with glucuronic acid or sulfate and are excreted in the bile as thyroxine glucuronide or triiodothyronine sulfate(28).

The portion of plasma iodide concentrated in the salivary and gastric glands undergoes intestinal recirculation. The remainder is excreted in the urine. These compounds are excreted in the feces or reabsorbed after hydrolysis of the conjugate by specific enzymes of enteric bacteria. All of the enterically excreted iodine is organically bound(27).

The hormones are also deiodinated and broken down in the liver and peripheral tissue to a variety of iodinated compounds of varying activity(28). T-3 and T-4 are deaminated to their pyruvic acid derivatives, which are decarboxylated and oxidized to acetic acid derivatives(28). In humans, the half-life of T-4 is 6 days and that of T-3 is 2 to 3 days(28).

C. Isotopes of Iodine

Standard man contains 15 principal elements, of which iodine is the least abundant, comprising 2.13×10^{-6}% of atoms(29). This amounts to 30 mg, most of which is in the thyroid(29). There are 29 radionuclides of iodine, which is double that of any other physiological element(30). I-127 is the only stable nuclide of iodine, and contains 53 protons and 74 neutrons. Myers(30) has classified the radionuclides of iodine as either neutron deficient or neutron excess.

The neutron deficient nuclides are produced mainly by positive ion accelerators, such as cyclotrons, while the neutron excess nuclides are produced in the neutron flux of fission reactors.

Of the 29 radionuclides of iodine, only I-123, I-125, I-131, and I-132 have been used clinically. Despite the wide use of Tc-99m, I-131 is still one of the most commonly employed radioisotopes in medicine. It can be seen from Table 1 (Nuclear Medicine Procedures with Radioiodine Labelled Compounds) that I-131 can tag a large number of different compounds with widely varying biological properties. For metabolic studies of the thyroid, the 8.05 day(29) half-life of this isotope is too long(29); for scanning procedures, the 364 KeV gamma emitted in 80% of disintegrations requires a thick NaI crystal with a heavy collimator(29). In addition, the high energy beta emissions cause unnecessary radiation doses, while high energy gamma emissions degrade the quality of scans(29). However, these factors combine to produce a satisfactory isotope for therapeutic treatment of the thyroid, in that a high thyroid radiation dose is produced, compared to the total body dose(29).

In recent years, cyclotrons which are especially designed and built for medical purposes are becoming more common in hospitals of large centers. These cyclotrons are capable of producing a large number of radioisotopes of biological and medical interest, including I-123. This radionuclide is the best nuclide of iodine for <u>in vivo</u> diagnostic applications(30), since it emits no beta particles and only a few low energy Auger and conversion electrons(30). Gamma rays of 159 KeV which have half thickness values of 4.7 cm in water, 0.37 cm in NaI and 0.037 cm in lead(30), are produced in 84 of every 100

TABLE 1

D. Nuclear Medicine Procedures with Radioiodine Labelled Compounds

Radiopharmaceutical	Administered Activity	Use
NaI-131	30 - 100 μCi	Thyroid scan
	2 - 10 μCi	Thyroid uptake test
		Treatment of hyperthyroidism
		Treatment of angina pectoris
		Treatment of thyroid carcinoma
		TSH studies
I-131 iodipamide		Blood pool scanning
		Joint Scanning
I-131 rose bengal	1.5 μCi/Kg	Liver and gall bladder scan
	1.0 μCi	Rose bengal excretion test
I-131 hippuran	0.5-3.0 μCi/Kg	Renogram
	0.5 μCi/Kg	Residual urine measurement
I-131 triolean	50 μCi	Triolean absorption test
	50 μCi	Oleic acid absorption test
I-131 Human Serum Albumin		Blood Pool Scan
	100 μCi	Isotope cisternography
	5 μCi	Placental localization
	8 - 12.5 μCi	Plasma volume
	8 - 12.5 μCi	Blood volume
	100 μCi	Mediastinal scan
		Circulation times
		Cardiac output
		Joint scan
I-131 Macro Aggregated Albumin	2 - 300 μCi	Lung scan
I-125 NaI		Thyroid scan
		Thyroid function
I-125 Human Serum Albumin	50 μCi	Protein losing gastro-enteropathy
	20 μCi	Blood volume
		Plasma volume
		Cardiac output
		Circulation studies
		T-3, T-4
		Radioimmunoassay of medically important compounds
I-123 NaI	10 - 100 μCi	Thyroid uptake and scan
		TSH suppression studies

disintegrations. These physical characteristics, combined with a half-life of 13.3 hours will deliver radiation doses which are only a few percent of those produced by a comparable amount of I-131. A summary of the physical characteristics of the different useful radioiodines is given in Table 2.

Iodine-132, which has a short half-life of 2.3 hours, had been advocated for nuclear medicine procedures in pregnancy and in children. This radioisotope can be obtained from a Te-132 ($t_{\frac{1}{2}}$ = 3.2 days) generator system. However, the emitted gammas and betas are too hard to be clinically useful(30).

A radioiodine scan of the thyroid with I-125 results in 50% of the radiation dose of an equal quantity of I-131(31). Blood volume determinations, however, result in a dose 1.4 times greater than those of I-131(31). For organs near the surface of the body, the use of I-125 will produce higher resolution scans, and will permit the use of light weight collimators. Contact autoratiograms can be made with I-125, which are comparable in detail to those made with tritium, and thus will permit the localization of the label at the cellular level(31).

I-125 has ideal properties for <u>in vitro</u> use. It has a long physical half-life of 60.0 days, which means that labelled compounds have a long shelf life(30). The gamma and x-ray emissions are energetic enough to prevent self-adsorption in water and other samples, and can penetrate counting vials and the aluminum shielding of crystals without appreciable loss(31). On the other hand, the radiation is low enough to be shielded by 1/16" of lead(30). Iodine has a relativ-

TABLE 2

E. Isotopes of Iodine: Advantages and Disadvantages(29,30,32)

Neutron Deficient Nuclides	Advantages	Disadvantages
I-123	1. short half-life(13.3 h) 2. gamma energy good for scanning (159 KeV) 3. no beta component 4. low radiation dose 5. useful in children and pregnancy 6. easy disposal, easy handling spills 7. excellent shielding 8. sp. act. up to 1900 Ci/mg	1. high cost 2. cyclotron production 3. radionuclidic purity: contaminated with I-124, 126, 130, 131 4. 3% disintegrations yield high energy gammas
I-125	1. long $t_{\frac{1}{2}}$(60.0 d) 2. long shelf life of labelled compounds 3. low radiation dose 4. light weight detection apparatus 5. good resolution of scans, radiograms 6. 1.4 photons/disintegration 7. sp. act. 17 Ci/mg	1. low energy gammas and x-rays attenuated by overlying tissue 2. high radiation dose <u>in vivo</u>

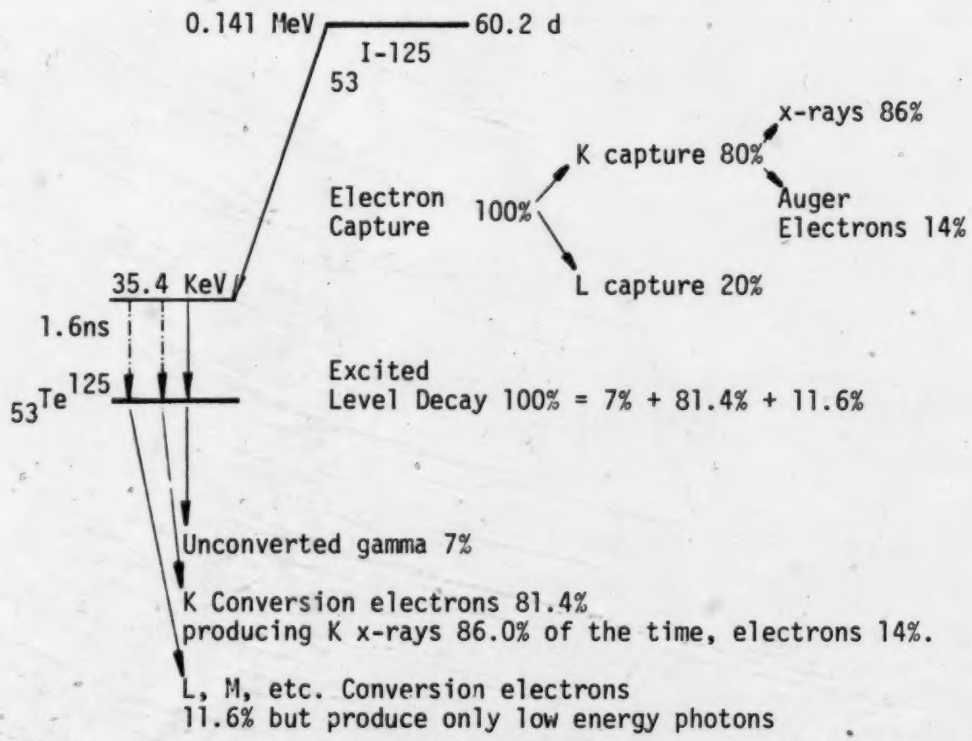
TABLE 2

E. Isotopes of Iodine: Advantages and Disadvantages(29,30,32)

Neutron Excess Nuclides	Advantages	Disadvantages
I-128	1. useful for N activation analysis in quantitation of I-127 2. short $t_{\frac{1}{2}}$ (25 min.)	1. gammas too hard for clinical use
I-130	1. Original radioisotope of iodine used clinically in 1938 2. short half-life (12.3 h)	1. hard gamma radiation 2. high energy betas
I-131	1. 364 KeV gamma 80% disintegrations for external scanning 2. high energy betas useful for therapy of thyroid 3. inexpensive	1. high radiation dose 2. long $t_{\frac{1}{2}}$(8.05 days) 3. high beta content 4. requires heavy detectors 5. high energy gammas give low resolution scans
I-132	1. short $t_{\frac{1}{2}}$(2.3h) 2. low radiation dose 3. generator product(Te-132) 4. useful in children, pregnancy, numerous studies at short intervals	1. expensive 2. requires proximity to source 3. very hard gammas and high energy betas

TABLE 3

F. Decay Scheme for I-125(35,36)



% of 27 KeV x-rays resulting from K capture is product of probabilities = 0.80 x 0.86 = 0.688 = 68.8%

% of 27 KeV x-rays resulting from K conversion electrons is product of probabilities = 0.814 x .86 = 0.70

% of detectable photons occurring from gammas and associated conversion electrons is sum of probabilities = 0.07 + 0.70 = 0.77 = 77.0%

% of detectable photons occurring per hundred disintegrations of I-125 is sum of probabilities = 0.07 + 0.70 + 0.688 = 1.458 = 145.8%

TABLE 3

F. Decay Scheme for I-125(continued)

% of time in which gammas with conversion electron x-rays occur simultaneously with K-capture x-rays is product of probabilities = 0.77 x 0.688 = 0.53 =	53.0%
% of time in which no gammas or x-rays will occur is the product of the probabilities that they will not occur: (100% - 68.8% = 31.2%) and (100% - 77% = 23%) hence, 0.312 x 0.23 = 0.072 =	7.2%
Percent of time in which K capture x-rays occur alone is the probability of its occurrence times the probability that gamma and conversion electron x-rays do not occur: 0.688 x 0.23 = 0.158 = 15.8%	
% of time in which gamma and conversion electron x-rays occur alone is the probability of its occurrence times the probability of K capture x-ray not occurring: 0.77 x 0.312 = 0.24 = 24.0%	
% of time in which gamma with conversion electron x-rays and K-capture x-rays occur in the absence of each other is the sum of the probabilities: 0.158 + 0.24 = 0.398 =	39.8%

ely simple chemistry and produces stable bonds with a large number of compounds that contain unsaturated bonds. The absolute disintegration rate of I-125 can be determined by counting only the singles and coincidence peaks. A summary of the radiation emitted from I-125 is given in Table 3.

In summary, I-125 decays by electron capture 100% of the time, of which 80% is K-capture, and 20% is L, M or higher electron capture. The product of this decay is the excited state of Te-125, which decays to the ground state by three processes: unconverted gamma emission 7%, K conversion electrons 81.4%, and L, M or higher electron conversion 11.6% (7% + 81.4% + 11.6% = 100%).

These two decay processes may occur alone (singles events) 39.8%, simultaneously (coincidence events) 53.0%, and with no detectable event 7.2% (39.8% + 53.0% + 7.2% = 100%).

G. Absolute Determination of I-125

Some radioisotopes decay by the coincident emission of two gamma rays. The measurement of the ratio of the number of gamma ray events which occur alone to the number which occur simultaneously allows one to calculate the absolute decay rate of that isotope(35). This may be achieved by use of a sodium iodide crystal with a single channel pulse height analyzer to measure the number of gamma-ray photoelectric interactions which occur alone (singles or non-coincidence peak) and the number of gamma-ray photoelectric interactions which occur simultaneously (sum or coincidence peak)(35).

The equation for the calculation of the absolute decay rate from the singles and coincidence counting rates has been derived in a

rigorous manner by Horrocks(35). This equation is:

$$N^\circ = \frac{P_1 P_2}{(P_1 + P_2)^2} \frac{(A_1 + A_2)^2}{A_2}$$

N° = absolute disintegrate rate
P_1 = emission probability for $gamma_1$
P_2 = emission probability for $gamma_2$
A_1^2 = singles count rate
A_2^1 = coincidence count rate

The probability of emission of a 35.4 KeV gamma-ray with 27 KeV K conversion electron x-ray was calculated to be 0.77 (77%), while the probability of a K electron capture 27 KeV x-ray was calculated to 0.688 (68.8%). The ratio of these probabilities is 0.688/0.77 = 0.89, which is approximately equal to unity. In other words, I-125 disintegrates as if it were emitting two independent gamma rays. The above equation then reduces to (35).

$$N^\circ = \frac{1}{4} \frac{(A_1 + A_2)^2}{A_2}$$

H. The Chemistry of Iodine

1. The Reactions of Iodine with Water:

Since all reactions of iodine of a biochemical nature occur in water, the reactions of iodine in and with water are of the utmost importance. The following reactions and rate constants are relevant to iodination of aqueous media:

1. Ionization of iodine

$$I_2 \rightleftharpoons I^+ + I^- \qquad K_1 = 10^{-20}(24)$$

2. Dissociation of triiodide

$I_3^- \rightleftharpoons I_2 + I^-$ $K_2 = 1.38 \times 10^{-3}$(38)

3. Hydrolysis of iodine to form hypoiodous acid

$I_2 + H_2O \rightleftharpoons HIO + H^+ + I^-$ $K_3 = 5.4 \times 10^{-13}$(38)

4. Hydrolytic ionization of iodine

$I_2 + H_2O \rightleftharpoons H_2OI^+ + I^-$ $K_4 = 1.2 \times 10^{-11}$(38)

5. Hydrolysis of H_2OI^+ and I^+

$H_2OI^+ + H_2O \rightleftharpoons HOI + H_3O^+$ $K_5 = 3 \times 10^{-2}$(24)

$I^+ + H_2O \rightleftharpoons HOI + H^+$

6. Dissociation of Hypoiodous acid

$IOH \rightleftharpoons H^+ + IO^-$ $K_6 = 1 \times 10^{-11}$(37)

7. Hydrolysis of hypoiodous acid

$HIO + 2H_2O \rightleftharpoons IO_3^- + 5H^+$

8. Formation of hypoiodite from iodine in alkaline solution

$I_2 + 2OH^- \rightleftharpoons I^- + IO^- + H_2O$ $K_8 = 30$(26)

9. Disproportionation of hypoiodite to form iodate

$3\ IO^- \rightleftharpoons 2I^- + IO_3^-$ $K_9 = 10^{20}$(26)

10. Iodate Formation from Iodine

$3I_2 + 3H_2O \rightleftharpoons IO_3^- + 5I^- + 6H^+$ $K_{10} = 4 \times 10^{-48}$(24)

11. Iodine Monochloride Formation

$I^+ + Cl^- \rightleftharpoons ICl$

12. Dissociation of Iodine Monochloride with excess chloride

$I_2Cl^-(aq) \rightleftharpoons I_2\ (aq) + Cl^-\ (aq)$ $K_{12} = 6 \times 10^{-1}$(24)

13. Hydrolysis of iodine monochloride

$ICl + H_2O \rightleftharpoons HOI + HCl$

$ICl + H_2O \rightleftharpoons H_2OI^+ + Cl^-$ $K_{13} = 10^{-4}$(41)

14. Iodine Formation from Iodine Monochloride(26)

$$5ICl + 3H_2O \rightleftharpoons IO_3^- + 5HC_l + 2I_2 + H^+$$

15. Equilibria of Iodine Monochloride in Water

$$ICl_2^- \rightleftharpoons ICl(aq) + Cl^- \qquad K_{15} = 6 \times 10^{-3}(24)$$

$$2\ ICl(aq) \rightleftharpoons I_2(aq) + Cl_2(aq) \qquad K_{16} = 1.6 \times 10^{-10}(24)$$

The interactions of iodine and water were examined by Hughes(37). based on the assumption that the tri-iodide method of iodinating proteins was the method of choice. From equation 1, I^+ cannot exist in significant concentration in water, so I^- is required to bring I_2 into solution (equation 2). From equation 3, Hughes(37) concluded that I_3^- slowed the reaction rate by decreasing the activity of iodine. Aromatic electrophilic substitution with iodine also produces an iodide anion, which has the same effect.

Hydrolytic ionization of iodine (equation 4) also yields another anion, so that the rate of formation of H_2OI^+ from tri-iodide is dependent on the inverse square of the iodide concentration, and therefore so is the reaction rates involving this species(41).

$$[H_2OI^+] = K_2\ K_4\ [I_3^-] / [I^-]^2$$

The hydrated cation H_2OI^+ and the free cation I^+ form HOI (equation 5); HOI also exists in equilibrium according to equations 6 and 7. At low concentrations of iodide, the formation of I_2 is favoured (equation 2), as well as H_2OI^+ and I^+ (equations 1 and 4)(41). According to equation 10, iodate formation should be extremely dependent on iodide concentration. In view of equation 8, Hughes(37) has suggested that pH 10 is the practical upper limit for iodinating proteins.

At the typically low concentrations of iodine used for protein iodination in buffered saline, the formation of electrophilic iodine will probably result in the formation of ICl, since the Cl^- ion will be present in a very much greater concentration (equations 11 and 12) (40, 41). The ICl so formed will react with water (equation 13) to form H_2OI^+, which will be in greater concentration than if it were formed from iodine. However, ICl can also generate I_2 from water (equation 14)(41,39).

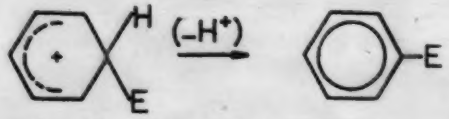
2. Iodine and Electrophilic Aromatic Substitution:

Common electrophilic aromatic substitution reactions involving such electrophiles as nitrate, sulfonate, and the halogens follow four steps according to Gutsche and Pasto(42):

1. The formation of the electrophile E^+.
2. Association of the electrophile (a Lewis acid) with the aromatic ring (a Lewis base) to form the pi complex in which the electrophile is loosely associated with the aromatic ring.

3. Rearrangement of the pi complex to a sigma complex or benzenonium ion, in which the electrophile becomes associated with a specific carbon atom.

4. Regeneration of the aromatic system by explusion of a proton.



In aromatic chlorination and bromination, the electrophile has been shown to be Cl_2 and Br_2(41). The iodine electrophile has been a subject of controversy.

Cofeman(43) studied iodination of phenol by ICl and concluded that the iodinating species was HOI. Painter and Soper(44) found that the rate of the reaction between $I_2$and phenol varied inversely with hydrogen ion concentration and the square of the iodide concentration. They also concluded that the electrophile was HOI and unionized phenol or I^+ and phenozide ions, and that there was acid catalysis. Berliner(41) reported that in the iodination of aniline and phenol, I_2, I_3^-, I^-, HOI, OI^-, and I^+ were possible electrophiles. I^- and I_3^- were ruled out because of the inverse dependence of the reaction rate on the iodide concentration. The rate of iodination of phenol was greater than that for aniline, and since aromatic ring activation by substituents is $R\text{-}O^- > R\text{-}NH_2 > R\text{-}OH$, it was suggested that phenol iodination proceeds by phenolate anion(41).

HOI was eliminated as the electrophile by a study of the kinetics of aniline iodination. Berliner(41) compared the kinetics of the reactions represented by the following rate equations:

$$-d[I_2]/dt = k[C_6H_5NH_3^+]\,[HOI]$$

$$-d[I_2]/dt = k'[C_6H_5NH_2]\,[H_2OI^+]$$

It was proposed that H_2OI^+ was the iodinating agent because the anilinium ion should yield meta substituted aniline, but only ortho, para substitution was produced. By analogy, Berliner(41) suggested

that the proton was on the hypoiodous acid, rather than on the phenoxide ion. The data are consistent with the following reaction mechanism(41):

$$I_3^- \overset{K_2}{\rightleftharpoons} I^- + I_2$$

$$I_2 + H_2O \overset{K_1}{\rightleftharpoons} H_2OI^+ + I^-$$

$$H_2OI^+ + ArH \underset{k-1}{\overset{k1}{\rightleftharpoons}} ArHI^+ + H_2O$$

$$ArHI^+ \overset{k2}{\rightleftharpoons} ArI + H^+$$

Since $[H_2OI^+] = K_1K_2[I_3^-] / [I^-]^2$ the observed rate constant becomes(41):

$$k_{obs} = k_1K_2K_1 / [I^-]^2$$

in which the rate depends on the inverse square of the iodide concentration.

However, the observed rate constant is also consistent with the possibility that I_2 is the electrophile. This was proposed when it was observed by Groverstein and Kilby(45) that phenol iodinates at a rate four times that of 2,4,6-trideuterophenol. This suggests that deprotonation is the rate limiting step in the following mechanism(41):

$$ArH + I_2 \underset{k-1}{\overset{k_1}{\rightleftharpoons}} ArHI + I^-$$

$$ArHI^+ \underset{k_2}{\rightleftharpoons} H^+ + ArI$$

Berliner(41) suggested that since another iodide ion is formed the observed rate equation is

$$k_{obs} = k_1k_2K_2/k_{-1}[I^-]^2$$

and is also dependent on the inverse square of the iodide concentration. At low concentrations of iodide $k_1[I^-]$ approaches zero and

the reaction rate is proportional to the inverse 1st power of the iodide concentration. This was observed by Groverstein and Aprahamian(46) in the iodination of p-nitrophenol.

The isotope effect depends on the partitioning of the intermediate between the reactants and the products. In the case of phenol, the intermediate may be especially stable, due to the loss of a proton(41).

Intermediate partitioning depends on the ratio of $k\text{-}1[I^-]/k_2$. If k_2 is much greater than $k\text{-}1[I^-]$, little of the intermediate reverts, and k-1 is rate controlling; if $k\text{-}1[I^-]$ is much greater than k_2, most of the intermediate reverts to reactants and k_2, the rate constant for deprotonation is rate controlling(41). It was also observed by Groverstein and Aprahamian(46) that the isotope effect decreased at low iodide concentrations.

Therefore, a cautious conclusion has been drawn by Berliner (41) that I_2 is the electrophile in aqueous iodinations. However, the electrophile may change with the substrate and iodide concentration. It has been reported in a number of papers on the iodination of different aromatic substrates, including tyrosine and histidine, that the observed rate of iodination can be represented by two terms of different iodide ion dependence(41):

$$k_{obs} = A/[I^-] + B/[I^-]^2$$

The first term represents iodination by I_2 and the second by HOI or H_2OI^+, which would be important at low iodide concentrations. Thus, it remains to be resolved if the species H_2OI^+ is involved in

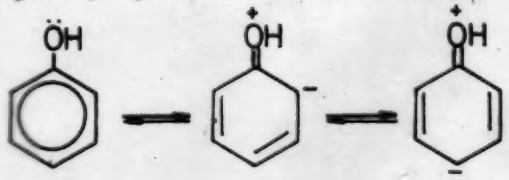
iodination.

3. The Reactions of Iodine with Amino Acids:

In biological systems, iodine undergoes three types of reactions: addition, substitution, and oxidation(24). Additions occur to unsaturated fatty acids and their esters, but there has been no clear demonstration of addition to amino acids. The oxidative reactions are undesirable side reactions in the iodination of proteins. Substitution reactions form stable derivatives with amino acids and are consequently the most useful.

4. Iodination of Tyrosine:

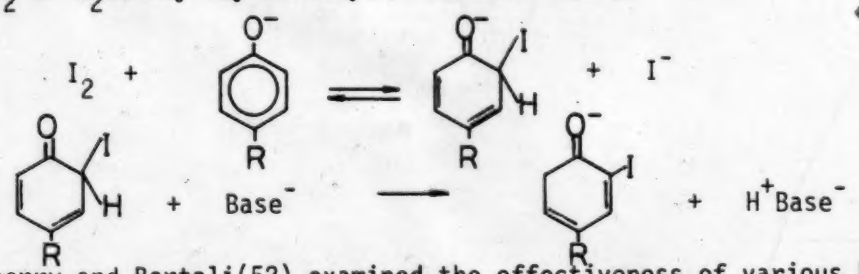
The hydroxyl function of phenols interacts with the benzene ring, so that there is a greater electron density in the aromatic ring than in benzene. The electron density accumulates at positions ortho and para to the hydroxyl group, thus it is activating, ortho-para directing(42).



A study of the kinetics of iodination of tyrosine by Li suggested that mono-iodination was the rate limiting step in the formation of diiodotyrosine (DIT). Fink et al.(48) and also Roche et al.(49) found monoiodo-tyrosine (MIT) in iodinated proteins. Mayberry et al. (50) studied the iodination of N-acetyl-tyrosine. At constant pH and I^- concentration, the reactions followed a bimolecular second order reaction rate.

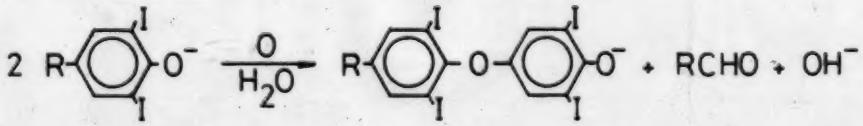
$$dx/dt = [A][B]$$

where x is mono or di-iodo N-acetyl tyrosine, and A is N-acetyl-tyrosine or N-acetyl-monoiodotyrosine and B is iodine(50). The rate increased with increasing pH, because of the increased concentration of the phenoxide ion(45). Over the pH range of 5.4 to 9.8, monoiodination was thirty times faster than diiodination(50). This was considered by Mayberry to be caused by the inductive quality of the iodine atom on the ring(50). Over pH 9.8, the iodate formation became significant as predicted by Hughes(37). The results supported a mechanism of a base catalyzed iodination of a phenoxide ion either by I_2 or H_2OI^+ by way of a cyclohexadienone(51).



Mayberry and Bertoli(52) examined the effectiveness of various buffers on the same reactions, over the same pH range. The effectiveness of the bases as catalysts in decreasing order was: $OH^- > CO_3^{2-} > HPO_4^{2-} >$ barbiturate $> CH_3COO^- > HCO_3^- > H_2PO_4 > H_2O$

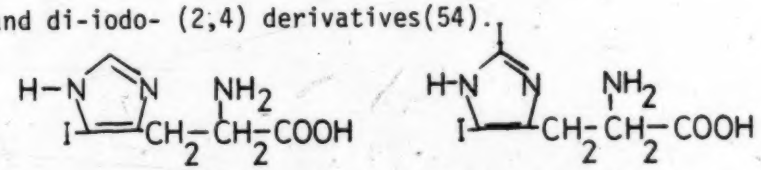
Edelhoch et al.(53) reported that thyroxine formation occurs only in highly iodinated proteins, i.e., at iodine to protein ratios (I/P) above 25:1. Thyroxine is formed as follows:



5. Iodination of Histidine:

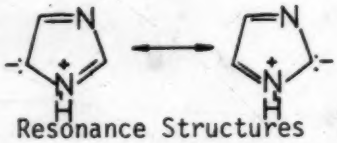
Histidine is ∝-amino-4 (or 5)-imidazole-propionic acid(25). It is an essential amino acid for most animals, but it has been

reported that is not essential for humans(54). It forms mono- (4) and di-iodo- (2,4) derivatives(54).



The classical structure of the imidazole heterocyclic ring of histidine is not consistent with its aromatic behaviour, tautomerism, and high dipole moment(55). It is best represented by a mesomeric structure or a set of resonance structures, including dipolar structures(55).

Mesomeric Structure

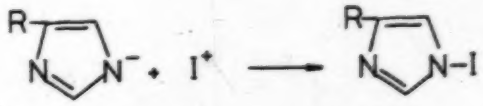


Resonance Structures

The contributions of ionic resonance structures of imidazole are more important than the contribution of ionic structures to the benzene ring. As a result, the imidazole ring possesses increased reactivity. Electrophilic reagents attack the lone electron pair on the multiply bonded, basic pyridine nitrogen, but not the weakly acidic pyrrole nitrogen(55). The carbon atoms of the ring are attacked by electrophilic, nucleophilic and rarely free radical reagents(55). Imidazole is aromatic in nature because of the sextet of p-orbital electrons which are contributed to the pi clouds. Each methylene carbon contributes 1, the pyridine nitrogen contributes 1 (the 3rd sp^2 orbital contains the lone pair of electrons) and the pyrrole nitrogen contributes two(54).

Total electron density calculations predict that electrophilic attack should occur preferentially at carbon 4(55) of the imidazole

ring. Iodination of imidazole occurs initially at C-4, forming 2,4,5-tri-iodoimidazole(55). It has been discovered that N-halo compounds are intermediates in this reaction. Halogenation involves the conjugate base of imidazole and iodination involves the imidazole anion at pH 7.0. It occurs initially at the 4 or 5 positions, probably *via* the N-iodo compound. Iodination follows the values of localization energies and depends on the transition state of the reaction, with the proton removal as the rate limiting step(55). The following reaction shows the formation of the N-iodo derivative:



Wolff *et al.*(56) reported that derivatives in which an imidazole nitrogen was substituted resisted iodination, whereas 2 or 4 histidine derivatives yielded iodinated products. It was also found that there was an increased yield of iodination with increasing pH. This was due to the first order dependence of iodination on anion concentration (56).

Although it has been suggested that N-iodo compounds are intermediates because N-methyl derivatives fail to iodinate, Wolff has found that the 2-t-butyl imidazole derivative in which the t-butyl function blocks access of iodine to N-1, does in fact, form an iodinated derivative, suggesting that only anion formation is necessary.

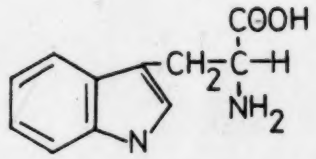
Schutte et al.(57) have investigated base catalysis in aromatic iodination, including p-cresol and imadazole, using various bases as a catalyst. For the reactions showing an isotope effect, including phenols and imidazole, the catalysis followed the nucleophilicities of the base rather than their basicities(57). This may be due to the fact that in these reactions, the rate limiting step consists of the nucleophilic attack of the base on the proton being abstracted(57). A mechanism was proposed by Schutte for base catalyzed aromatic iodination, from which he developed a kinetic equation which included the phosphate buffer (or water)(58).

The reactivity of the dissociated imidazole ring is 10^8 times greater than the neutral ring(58). In histidine and monoiodohistidine, the ammonium group in the side chain forms a hydrogen bond with the imidazole nucleus, which will decrease reactivity because of its deactivating influence on the imidazole anion(58).

Schutte et al.(58) reported a specific function of phosphate ions in aromatic iodination in which phosphate ions rupture and compensate for the intramolecular hydrogen bond or open a more effective path of iodination. A catalytic function of phosphate in aromatic iodination was also observed by Li(59) and by Vaughn et al. (60).

6. Iodine Reaction with Tryptophan:

Tryptophan is l-α-aminoindole-3-propionic acid and is classified as essential for growth in rats and is not synthesized by humans(25).



It has been known for some time that tryptophan does not form a stable bond with iodine, but rather undergoes degradation(61). Alexander(62) has found that both chemical and enzymatic iodinating agents rapidly oxidize tryptophan to 2-oxindole over the pH ranges 2 to 6 and 9.5 to 10.5, with a maximum at 4.5 to 5.5. In acid pH, this oxidation leads to cleavage of tryptophanyl peptide bonds, whereas in alkaline media (6.5 to 8.5) less fission occurs(62). Alexander found that I_2 and ICl oxidize N-benzyloxycarbonyl-tryptophanylglycine (Z-tryp-gly) 1.5 to 2.0 times more effectively than tri-iodide. Three equivalents of tri-iodide disappeared per mole of tryptophan oxidized, which is the same as oxidation by bromine. The peptide was also oxidized to oxindole by chloramine T, N-iodosuccinamide, lactoperoxidase and horse-radish peroxidase(57). ICl, chloramine T - KI, N-iodosuccunamide completely oxidized tryptophan at pH 5.0 within 1 minute, but I_2 and tri-iodide were 2 and 8 times slower respectively(62). Oxidation of tryptophan is to be expected because of the reactivity of indole to electrophilic reagents.

Oxidative cleavage also occured at an acid pH, but at a lower rate than oxidation to oxindole. The maximum occurred at pH 4.5-5.5 with no fission occuring in alkaline media(62). Cleavage is promoted at a rate proportional to oxidation potential of the iodinating agent. No significant hydrolysis occurs above pH 7.

Alexander has proposed a reaction mechanism for oxidation and oxidative cleavage of Z-tryp-gly during iodination, which follows that found for brominating agents(62). Competition experiments with equimolar amounts of Z-tryp-gly and Z-try-gly suggested that tryptophan oxidation occurs selectively at pH 5.0, but oxidation at pH 7.5 was reduced by 50% or more because tyrosine was more effectively iodinated at high pH , but Z-Met-Gly and Z-His-Gly competed less effectively at either pH(62).

Alexander(62) found that the factors that affect tryptophan oxidation during protein iodination were pH, iodinating reagent and concentration, tryptophan and tyrosine content, accessibility of reactive residues, and the nature of the protein(62).

7. Iodine Reaction with Cysteine:

According to Hughes(37), the reaction of iodine and sulfhydryl is:

$$R\text{-}SH + I_2 \longrightarrow RSI + HI$$

Khalkhali et al.(63) concluded that sulfhydryl iodide may react with another sulfhydryl or hydrolyze and oxidize with molecular oxygen.

$$R\text{-}SI + RSH \longrightarrow R\text{-}S\text{-}S\text{-}R + I^-$$

$$R\text{-}SI + H_2O \longrightarrow R\text{-}SOH + HI$$

$$R\text{-}SH \xrightarrow{O} R\text{-}SOH \xrightarrow{O} R\text{-}SO_2H \xrightarrow{O} R\text{-}SO_3H$$

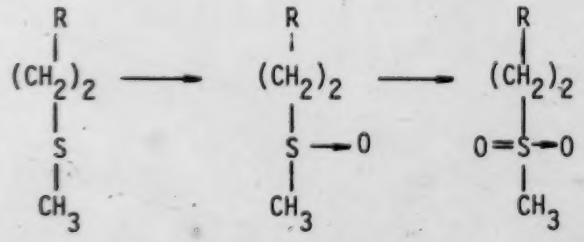
or $$R\text{-}SH + 3H_2O \longrightarrow R\text{-}SO_3H + 6H^+ + 6e^-$$

Since 6 electrons are produced, six Faradays(F) per mole are consumed without dimer formation. Simonsen(64) showed that when iodine oxidized low concentrations of cysteine, sulfenic (R-SOH) and sulfinic acid ($R\text{-}SO_2H$) derivatives are produced. The reports of Rosa et al.

(65) and of Munze et al.(66) suggest that the sulfonic acid derivative ($R\text{-}SO_3H$) may be produced, since 5.3 and 5.9 Faradays/equivalent of sulfhydryl were consumed. Khalkhali reported that 4 Faradays were consumed per mole of SH in albumin, 1.5 F/SH in gamma-globulin, 0.7 - 0.9 F/mole SH for transferrin, 3 F/mole of SH in fibrinogen(63).

8. Iodine Oxidation Reactions with Methionine and Hydroxyamino Acids:

Free methionine, with a thioether linkage R_2S, reacts to form a perhalide which hydrolyzes to form a sulfoxide or sulfoxone derivative(67). Koshland(67) iodinated antibody to the extent of 100 iodine atoms per protein molecule (I/P ratio) and found that iodination oxidized methionyl, tryptophanyl and cysteinyl residues. Morely(68) has shown that the methionyl residue at position 15 in gastrin is readily oxidized to sulfoxide and sulfone derivatives.



Ramachandran(69) observed that the hydroxyamino acids might also be expected to be oxidized to carbonyl derivatives, but Koshland (67) found that they did not react. Phenylalanyl and cystinyl residues also did not react(67).

9. Iodine Reactions with Proteins:

Iodine has poor selectivity in its reactions with proteins

and the reactions are highly dependent on reaction conditions. The functional groups may be arranged in order of decreasing reactivity with iodine: sulfhydryl, phenol, imidazole, indole, sulfide, disulfide(70). Similarly, reactive iodine species can be arranged in order of decreasing oxidative ability: N-iodosuccinimide > ICl = (Chloramine T-KI) > Chloramine T > I_2 = peroxidase > I_3^-. The oxidative potential is highly pH dependent(61,62).

Li(47) studied the iodination of albumin and pepsin and noted that not all tyrosine residues were reactive. After partially denaturing the proteins in urea, more groups were reactive and DIT formation was increased(47). After total denaturation, all groups were reactive. Hughes and Straessle(71) suggested that several reactions of iodine with albumin proceeded simultaneously, including the iodination of histidine.

Covelli and Wolff(72,73) found that iodination of lysozyme in water produced two iodinated tyrosyl residues and a third was iodinated in 8 M urea. Monoiodotyrosine was the major product at less than two moles of iodine per mole of protein, while diiodotyrosine was produced at values greater than that ratio. Iodinated histidine was produced only at high molar ratios, and T-4 was not observed. They found from the iodination of ribonuclease A that a single iodinated histidyl residue was produced only after the ortho positions of three reactive tyrosyl residues had been iodinated(74), and similarly, the two reactive histidyl residues of insulin were iodinated after the four reactive tyrosyl groups were derivatized(75).

Wolff and Covelli(76) described the factors that influenced the

reactivity of iodine with histidyl in proteins, the most important of which was the direct dependence on pH. Diiodohistidine was formed mainly in proteins deficient in tryptophan.

Edelhoch et al.(53,77,78) in a series of papers reported the quantities of tyrosine, MIT, DIT, and T-4 in thyroglobulin, gamma globulin, and albumin produced when iodinated with increasing amounts of iodine. The following trends were noted: when less than 10 moles of I_2/mole of protein were used, MIT was the major product. As the I/P ratio was increased to 50:1, the non-iodinated tyrosyl residues decreased, whereas DIT and T-4 increased. One percent of the total phenolic residues converted to T-4 in albumin and globulin at iodine to protein rations of ca. 25:1, and 5%, 2% and 4% were converted in thyroglobulin, globulin, and albumin at a ratio ca. 50:1. They concluded that MIT is preferentially converted to DIT when tyrosyl residues are located in a hydrophobic atmosphere.

Koshland(67) observed that in heavily iodinated antibody (100 I /molecule of protein) iodine was substituted in tyrosine and histidine, and oxidized methionine, tryptophan, and cysteine while phenylalanine, serine, threonine and cysteine did not react. Krohn et al.(79) reported the following yields of labelled derivatives after labelling fibrinogen at an I/P ratio of 1/1: 78.3% MIT,5.2% DIT, 1.5% MIH and 0.7% DIH. At a ratio of 25:1, the yields were 57.8% MIT, 21.0% DIT, and 2.5% MIH.

The number of free sulfhydryl groups in proteins is generally low. Khalkhali(63) reported that albumin contained 1 per molecule, gamma globulin 0.7 per molecule, fibrinogen 0.5 per molecule, and

transferrin had none. Rosa(65) suggested that oxidized and non-oxidized sulfhydryl groups may co-exist when iodination occurs at low levels. Stable sulfenyl iodide has been observed in the iodination of ovalbumin, and B-lactoglobulin and tobacco mosaic virus protein (70). In a study of various iodinated proteins, Cunningham et al.(80) concluded that intermolecular disulfide formation does not occur and intramolecular formation is not a major factor. The work of Cohen(70) suggests that complete oxidation of sulfhydryl residues appears to have no influence on metabolic clearance.

Hung(81) reported his findings regarding the pH conditions most suited to the highest rate of aromatic substitution and for the slowest rate of oxidation of disulfide bridges. Tyrosine (1 mM) was reacted with ICl (1 mM) at pH 4.0, and it was found that the reaction occurred in 12 seconds, while histidine first catalyzed the hydrolysis of ICl to I_2, after which I_2 reacted slowly with cationic histidine(81). Most histidine containing peptides acted similarly. At pH 7.4 and pH 9.5 both tyrosine and histidine reacted rapidly with ICl. The alterations produced by ICl to several sulfur containing compounds including L-cysteine was measured by circular dichroism at pH 2, 7 and 8.5. It was found that the least alteration was produced at pH 7.0. Hung(81) also concluded that it was most favourable to carry out iodinations by successive additions of small aliquots of ICl.

Oncely(82) has observed that in the iodination of protein molecules containing a number of tyrosyl residues, the distribution of iodine among tyrosyl residues and between protein molecules would

be uneven. Molecules with the same number of iodine atoms would differ from each other in the distribution of iodine among the tyrosyl residues. He has proposed a binomial distribution of iodine atoms among the protein molecules(82):

$$\frac{nCn}{\Sigma nCn} = \frac{m!n}{n!(m - n)!R}(R/m)^n(1 - R/m)^{m - n}$$

where:

nCn	-	radioactivity of derivative containing n groups
ΣnCn	-	total radioactivity where n varies from 0 to m
m	-	maximum number of reactable residues
n	-	number of residues reacted
R	-	ratio of iodine to protein
Cn	-	concentration of derivative containing n groups

II. METHODS OF IODINATING PROTEINS

The various procedures of iodinating proteins are merely different methods of generating the electrophile I^+. This has been achieved by four general methods:

1. tri-iodide ion or iodine
2. chemical oxidation of I^-
3. enzymatic oxidation of I^-
4. electrochemical oxidation of I^-

A. Iodination by Triiodide Ion or Iodine

The triiodide method was considered by Hughes(37) as the method of choice, since the presence of the iodide ion buffered the reaction of iodine with the protein. The method, called the bicarbonate iodination method, used aqueous solutions of protein albumin buffered at pH 10 with bicarbonate. I_2 and I^- were mixed in a ratio of 1:2

with radioiodine and added dropwise with stirring to the protein solution. The reaction could be stopped by adding sulfite(71).

The method was used by Covelli and Wolff(76) in their study of iodination of tyrosine and histidine in lysozyme, ribonuclease, insulin, lactic, malic and glutamic hydrogenases, phosphoglucomutase, thyroglobulin, trypsin, insulin, chymotrypsin and B-lactoglobulin. They were iodinated at pH 8.5 in tris buffer with 0.046 M I_2 in 0.14 M KI. This method is still in current use as indicated by the report of Ishiguro et al.(83) who labelled Ricin D at pH 7.0 with 0.05 N iodine in 0.1 M KI.

Labelling with iodine itself is not encountered frequently. It was used by McFarlane before the introduction of ICl. Elemental radio-labelled iodine was prepared in a solution and injected at high velocity into a counterstream of glycine buffered protein at pH 9.3(84).

B. Iodination by Chemical Oxidation of I^-

1. Miscellaneous Methods:

A large number of chemical oxidation agents have been used to oxidize I^- to the electrophilic I^+, including nitrous acid(85), iodate, hypochlorite(86), hydrogen peroxide(87), persulfate(88), and chloramine-T(21).

In the ammoniacal iodination method of Frances(86), I^- is oxidized by iodate in the following equation:

$$5KI + KIO_3 + 6\,HCl \longrightarrow 6\,KCl + 3\,H_2O + 3I_2$$

The iodinating solution was added dropwise to a gamma globulin solution to which had been added a small quantity of 5N NH_4OH.

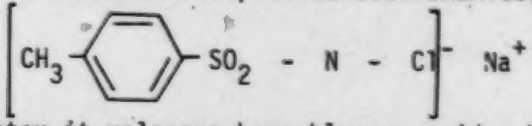
McFarlane(84) used hydrogren peroxide to liberate iodine from KI in a buffered solution of gamma globulin. Hemmings and Redshaw(87) used sodium hypochlorite (0.5-1.0 mM) to release iodine monochloride at pH 7.5.

$$HClO + I^- + H^+ \longrightarrow ICl + H_2O$$

Sodium hypochlorite was mixed with 400 µCi of I-125 and 2 µg of immunoglobulin for 30 seconds, and the reaction was stopped with sodium metabisulfite (5mg/ml).

2. The Chloramine-T Method:

The most commonly employed oxidizing agent is chloramine-T, which was introduced by Hunter and Greenwood(21). It is the sodium salt of N-monochloro-p-toluenesulfonamide(89).



In water it releases hypochlorous acid, which is the oxidizing agent.

$$R\text{-}NCl^- + H_2O \longrightarrow R\text{-}NH_2 + HClO$$

The procedure consists simply of adding the protein, and then the chloramine-T to a solution of sodium iodide, spiked with sodium radioiodide. The radioiodide is converted in the presence of stable I-127 to ICl and the reaction achieved a theoretical maximum of 100% substitution of iodine into the protein.

Yields of over 80% were achieved for iodination of human growth hormone (HGH)(89) with specific activities ranging from 200 to 750 µCi/µg. The ratio of substitution was as low as 0.5 to 1.0 atom of iodine per molecule of peptide with no detectable degradation products (21). Five µg of protein in 25 µl of pH 7.5 phosphate buffer was

added to 0.5 - 2 mCi of I-125 or I-121 in 25 µl of phosphate buffer. Freshly prepared chloramine-T (100 µg in 25 µl phosphate buffer) was added rapidly and mixed. Fresh sodium metabisulfite (240 µg/0.1 ml) and KI (2 mg/0.2 ml) were added to stop the reaction; it was found that if the reducing agent was added in excess, denaturation occurred(89).

In experiments with albumin, the ratio of chloramine-T to protein was found to be important. At an I/P ratio of 1:1, chloramine-T was used at a chloramine-T/iodine ratio of 1:1(89). At low concentrations of iodide, a chloramine-T/iodide ratio of 100:1 was required, and the ratio was increased with increasing specific activity(89). In general, the method was found useful for producing exceedingly high specific activities from minute quantities of hormone.

McConahey and Dixon(90) reported a modification of the chloramine-T method of Hunter and Greenwood in which they labelled µg or mg quantities of 50 proteins with little or no detectable denaturation. They added one-thousandth the amount of chloramine-T (100 µg) that was used by Hunter and Greenwood and increased the reaction time to 5 minutes before adding the sodium metabisulfite. The yields ranged from 40% to 90%(90).

Bocci(91) has made a study of labelling serum proteins (gamma globulin and albumin) and tissue-soluble proteins with I-131 and chloramine-T. He found that proteins labelled at a ratio of protein to chloramine-T less than 200:1 were unchanged in electrophoretic mobility, gel filtration profile, and chromatographic behaviour, whereas the metabolic fate was adversely modified in comparison to

proteins labelled by the iodine monochloride method(91).

Charkes et al.(92) used the chloramine-T method to produce 90-95% clottable I-131 fibrinogen. It was used to image deep venous thrombi in post-operative patients(92).

Lazewatsky and Murray(98) labelled bovine fibrinogen by the chloramine-T method. The chloramine-T to fibrinogen ratio was 13:1 with a large amount of fibrinogen which results in an iodine to protein ratio of 0.1. The reaction was stopped with $Na_2S_2O_4$. The supporting buffer was 0.1 M phosphate 0.15 M NaCl-4mM epsilon amino caproic acid. Isotopic clottability was 92.5% and the half-life in calves was 5.1 days(93).

Krohn, Metzger and Coleman(16,17,18,19) examined canine fibrinogen labelled by the chloramine-T method and reported their results in a series of papers. Protein (5-200 µg) was added to 50 µl of phosphate buffered pH 7.8 carrier free I-125 followed by 25 µl of freshly prepared chloramine-T(16). $NaHSO_3$ was omitted and I^- removed by chromatography. The ratio of chloramine T to tyrosine was varied from 150 to 0.03(16). This procedure resulted in a product which had an aggregate content as high as 80%(16). Spectroscopic clottability decreased from 97% to 90% and isotopic clottability was 90%(16). The percent of I-125 binding was approximately 93-95%. The rate of hydrolysis was 2.21%/day in plasma, 1.49%/day in albumin and 19% in 1 day in saline(16). Less than 20% of the label remained attached to the fibrinogen in vivo. The aggregated material was found to be removed rapidly by the spleen and liver(128). In vivo, the unbound iodide reached a maximum of 20-80% in 10-30 hours(128).

The longest half-life of canine fibrinogen was 60.9 hours, 18% of the injected dose being cleared with that half-life(131). The activity of chloramine-T-labelled fibrinogen in surgically induced thrombi was 23% of the precordial count, compared to 115% for ICl labelled fibrinogen(18). The ratio of activity of labelled fibrinogen in the thrombus compared to the blood was 2.7:1, while the ratio was 8.8:1 for ICl labelled fibrinogen(18).

Frisbie et al.(134) added I-125 and 10 µl of chloramine-T solution (chloramine-T: fibrinogen = 12) to 0.5 ml fibrinogen in phosphate buffer (0.1 M, pH 7.4) and mixed for 10 seconds. After 6 minutes, 12 ml of 30% saturated ammonium sulfate was added, and the solution was centrifuged for 10 minutes. The precipitate was dissolved in 2 ml of buffer and sterilized by passing it through a Swinnex filter unit(134).

Hawker and Hawker(135) added 200 µCi of I-125 and 50 µl of 200 mg% of chloramine-T solution to 0.75 ml of fibrinogen in sodium citrate pH 7.4 in an ice bath, followed after 60 seconds by 100 µl of 400 mg% sodium metabisulfite. After 3 minutes, 100 µl of 1.7% KI was added. One ml of 1:1 dilution of patient plasma and sodium citrate was layered on top of a column of G-25 (a 10ml syringe containing a sintered polyethylene disc to which was attached a filter unit and a bottle) followed by 1 ml of fibrinogen solution. The column was centrifuged at 60 g for 5 minutes and 1000 g for 5 minutes. The sterilized and iodide-free fibrinogen in the bottle was detached from the column and sealed(135).

Roberts et al.(136) added 10 µl of NaI carrier (6×10^{-10} moles), 10 µl of NaI-125 (200 µCi) and 10 µl of chloramine-T (12×10^{-7} M) to

1 ml of fibrinogen solution (8.6×10^{-9}M) in 0.1 M sodium phosphate pH 7.4 and 0.9% NaCl. These chemicals were allowed to react for 6 minutes before stopping the reaction by the addition of 2 ml of plasma(136).

3. The Iodine Monochloride Method

Many workers have successfully used the iodine monochloride method developed by McFarlane(94). McFarlane observed that the yield with iodine monochloride is double that obtained with iodination by iodine in alkaline solution. The following reaction shows that in alkaline solution 50% of iodide is lost to salt formation.

$$I_2 + NaOH \longrightarrow HOI + NaI$$

The original procedures consisted of adding carrier free NaI-131 to a solution of cold iodine monochloride, where radioactive iodide would exchange rapidly with stable iodide. This labelled iodine monochloride solution was injected into glycine buffer at pH 8.5, which would regenerate hypoiodous acid. The iodinating solution was then injected into the protein solution in glycine buffer at a pH not above pH 9.5(94). In this procedure, the iodine must pass through a volatile phase, and in addition, carrier iodide must be added so that stable iodine is added to the protein(84). The yields were from 50% to 90%, depending on the ratio of iodine to protein in the iodinating mixture(94).

McFarlane(20) found that proteins substituted with 6 or more atoms of iodine were eliminated more rapidly than C-14 labelled proteins but at 0.5 atom per molecule, albumin, globulin and fibrinogen behaved the same way as the C-14 labelled proteins. He also

observed that more than 20% of labelled rabbit fibrinogen with 2 to 3 atoms of iodine per molecule was catabolized rapidly(20).

Helmkamp and his group published a series of papers iodinating gamma globulin to high specific activity(99). The I-131 was adjusted to pH 8 with borate buffer and HCl; Na_2SO_3 was added to destroy hydrogen peroxide that was produced by the I-131(95). The excess Na_2SO_3 was destroyed by aeration(96). ICl was added and the solution was added rapidly to 4 mg quantities of protein at pH 8.0. The labelling efficiency was 60% and specific activity was 10 mCi/mg. They also reported that catalase could be used to destroy the hydrogen peroxide in the I-131 preparation(97).

Helmkamp(97) examined the disproportionation of ICl to Cl^- and HOI at high pH, and of the latter into I_2 and iodate.

$$3H_2O + 3ICl \longrightarrow 3H^+ + 3Cl^- + 3HOI$$

$$5HOI + OH^- \longrightarrow 2I_2 + IO_3^- + 3H_2O$$

$$2I_2 + 2OH^- \longrightarrow 2HOI + 2I^-$$

$$3HOI + 3OH^- \longrightarrow 2I^- + IO_3^- + 3H_2O$$

He observed that the ratio of total iodine in NaI-131 preparations to ICl is so great that a large fraction of the I-131 escapes introduction into the ICl and is therefore not available for iodination of the protein. This defect could be remedied by adding radioiodine to the KI solution before it was oxidized to ICl, as in the following procedure(98). To 0.34 ml of 0.0067 M KI solution was added 0.3 ml of NaI-131 and diluted with 0.5-1.0 ml of saturated NaCl and enough water to make 3 ml after adding an excess of 25 µg/ml of KIO_3 and 0.15 ml of 0.5-1.0 n HCl. To rabbit gamma

globulin in 2.0 ml of pH 8.0 borate buffer was added 0.5 ml of 1 N NaOH in 1.5 ml of pH 8 borate buffer. The iodinating solution was jetted into the protein solution and the free iodide was subsequently removed by ion exchange(98).

As has occured with chloramine-T method, there have been several modifications of the ICl method published, including those by Glover(100), Gill(101), and Reif(102). For example, in Rief's modification(102), the pH of the iodine monochloride solution was raised from pH 0.3 to pH 2. This reduced the amount of alkali required to neutralize the ICl to the physiological range. The neutralizing borate buffer was added to the protein solution, rather than to the ICl, because at alkaline pH, the ICl was rapidly destroyed. Thus, the ICl could be added slowly to the protein solution containing the radioiodine solution. This produced labelling efficiences of 70-90% for quantities of gamma globulin ranging from 1 mg to 1 g. It was found that the yield increased with an increase in ICl protein ratio; at a ICl peptide ratio of 16:1, the yield was 92.3%(102).

Bale et al.(103) published a report on the factors that influence the iodination yield of the iodine monochloride method. They suggested that the efficiency of iodination could be represented by(103)

$$E = \frac{\text{Wt of I in ICl}}{\text{Wt of } I^- + \text{Wt of I in ICl}} \times Y$$

where Y is the percent iodination by that weight of ICl in the absence of iodide. The result is that adding an increasing amount of I-131 to an ICl system causes a steadily decreasing percent of incorporation.

Thus there is an optimum amount of I-131 and a maximum specific activity(103).

Ceska et al.(104) studied the labelling of proteins by ICl using a mathematical model derived from the mass law equation of the isotopic exchange between I-125 formed, and the number of millicuries required for labelling to a desired extent. The two equations used in the study are(104):

$$N = \frac{S^\circ M^\circ_I \, m_s/M_s \, (n^\circ I/s + nI/s)100}{\left| \frac{n_{I/s}}{n^\circ I/s} - \frac{M^\circ_I}{M_I} (S^\circ/S - 1) \right| n_{ICl}}$$

$$\%125_{ICl} = \left| \frac{1}{1 + \frac{M^\circ I}{M_I} (S^\circ/S - 1) + \frac{S^\circ M^\circ_I \, n_{ICl}}{N}} \right| 100$$

where:

- N - amount of I-125 required for labelling (mCi)
- S° - specific activity of I-125 (17 mCi/μg)
- M°_I - Molecular weight of I-125
- m_s - weight of the substance to be labelled
- M_s - molecular weight of the substance to be labelled
- $n^\circ \frac{I}{s}$ - number of I-125 atoms to be incorporated per protein molecule
- $n \frac{I}{s}$ - number of I-127 atoms to be incorporated per protein molecule
- M_I - molecular weight of I-127
- S - specific activity of I-125 preparation received (mCi/μg)
- n_{ICl} - iodination efficiency
- $\%125_{ICl}$ - % of total ICl that is radioactive under equation 1 conditions

Krohn, Metzger and Coleman(16,19,79,131) published a series of papers on the properties of canine fibrinogen labelled with I-125 by the ICl method of McFarlane as modified by Samols and Williams and by

Helmkamp et al. A few microliters of I-125 were equilibrated with ICl in 0.4 ml of 2 M NaCl(16). The solution was added to an equal volume of borate buffer at pH 8.0. Borate buffer was added to fibrinogen to the same volume as ICl. The ICl mixture was jetted into the fibrinogen solution and agitated for 15 minutes(16).

These workers found that less than 1.0% was aggregated(16). About 74 $\pm$ 2% of I-125 was bound to the fibrinogen; the spectroscopic clottability decreased from 97% to 96% and the isotopic clottability was 89%. The hydrolysis rate was 2.4% per day in plasma and 9% in one day in saline. It required 35 to 50 hours for the ICl labelled preparation to reach a maximum of 20-57% of unbound iodide in vivo (131). The longest half-life in dogs was 40.5 hours and 34.3% of the injected dose was cleared with this half-life(131). The activity of ICl labelled fibrinogen in surgically induced thrombi in dogs was 115% of that of the precordial count rate(18). The ratio of the thrombus to blood activity in the thrombus was 8.8:1(18). When a labelled preparation was hydrolyzed to the amino acids by proteases, it was found that 75-80% of the label was present in the protein as MIT(79).

Hagen et al.(136) added 0.35 ml of radioiodine monochloride (3×10^{-4} M ICl_2^-, 4×10^{-4} M KIO_3, 0.015 M HCl and 2 M NaCl) to 2 ml of fibrinogen solution (0.1 M PO_4 buffer, pH 7.4, 0.05 M epsilon amino caproic acid and 0.38% trisodium citrate) and mixed for 3 minutes (137). Nonprotein bound radioiodine was removed by injection of the solution into a Hopkins double closure vial, which contained 4 ml of 1% human serum albumin and sodium metabisulfite. The solution was stirred for 5 minutes before withdrawing the protein solution through

2 gm of Dowex 1 x -4(137).

Peabody et al.(138) labelled a large quantity of fibrinogen with I-125. Fibrinogen (100 mg) in 15 ml of citrate-saline buffer pH 7.4 was mixed with 1.8 ml of 0.9 M glycine buffer pH 8.5 and chilled to 0°C(138). Separately, 1 mCi of I-125 in 1 - 1.5 ml of water was mixed with 0.3 ml of carrier iodine monochloride and chilled to 0°C(138). After 30 minutes of equilibration, 1.3 ml of 0.8 M NaCl - 0.8 M glycine buffer pH 9.5 at 0°C was added to I-125 labelled iodine monochloride and the combined solution was jetted into the fibrinogen solution. The mixture was incubated for 30 minutes at 0°C, before dialysis against 0.13 M sodium chloride - 0.02 M sodium citrate pH 7.5 at 4°C(138).

C. Iodination by Enzymatic Oxidation of Iodide

The hydroperoxidases are a group of hemoproteins which include the catalases and the peroxidases(105). The peroxidases are a group of enzymes which are found in fungi, bacteria, plants, and in mammalian tissues and secretions(106). The peroxidases all contain one ferriprotoporphyrin IX in the high spin state as the prosthetic group(106). They exhibit characteristic absorption spectra and are inhibited by iron-binding agents such as cyanide(106).

The peroxidases catalyze the transfer of electrons from donors (substrates) to hydrogen peroxide, reducing it to water(106). They are specific in requiring hydrogen peroxide as the oxidizing agent, but various substances may act as substrates or electron donors. Peroxidase forms a green compound with hydrogen peroxide known as Compound I, and is reduced by one electron to form red Compound II.

Compound II is then reduced by one electron to reform the peroxidase (106).

Crude mixtures of peroxidases and hydrogen peroxide were shown to catalyze the iodination of tyrosine, thyroglobulin and other proteins(24). Thomas and Hager(107) studied chloroperoxidase and suggested the enzyme was effective in two stages. I^- was oxidized to form I_2 and the enzyme catalyzed I_2, H_2O_2 and tyrosine to form the iodinated product. However, Nunez and Pommier(108) showed that horseradish peroxidase, which catalyzes the iodination of tyrosine in thyroglobulin by I^-, complexes with the active iodine species, which is probably I^+, and is transferred directly to the protein. The enzyme thus forms a stable complex with I^+ and this intermediate can be isolated. It has been tentatively identified as sulfenyl iodide (24). Nunez and Pommier(109) concluded that the enzyme has two identical sites which form complexes sequentially with I^- and thyroglobulin.

The work of Pommier et al.(110) suggested a model which predicts a second order dependence on iodide concentration and was consistent with a bimolecular reaction between $2I^-$ ions on the surface of the enzyme, the product being released after dimerization or dismutation of the oxidized species(110), according to the following equation:

$$\text{Peroxidase: } H_2O_2 \text{ complex} + 2e^- \rightleftharpoons E(2I^\circ) \rightleftharpoons EI_2 \rightleftharpoons E + I_2$$

The data are consistent with the presence of two sites for the substrate at the surface of the peroxidase. One site binds the iodide and the second binds the protein to be iodinated(110).

$$E{:}H_2O_2 + I^- + \text{protein-tyrosine} \rightleftharpoons E\left\langle {}^{I^\circ}_{\text{Tyrosine-protein}} \rightleftharpoons E + \text{protein -I}\right.$$

I_2 formation is favoured at acidic pH and protein iodination at more alkaline pH. The lower the iodide protein ratio, the lower the I_2 yield and the higher the rate of protein iodination(110).

Morrison(111) suggested that lactoperoxidase catalyzes the iodination of tyrosine, while horseradish peroxidase was an inefficient catalyst if at all. Lactoperoxidase was found to iodinate L-tyrosine faster than D-tyrosine, while the converse was found with horseradish peroxidase(111). Lactoperoxidase was also found to form an enzyme-substrate complex with iodinatable tyrosine. This was demonstrated by the fact that histidine, which is also catalytically iodinated by lactoperoxidase at a slower rate than tyrosine, competitively inhibits the iodination of tyrosine at a common binding site(111). Morrison also speculated that the enzyme-catalyzed iodination reaction was an electrophilic attack of the iodinium ion produced by the peroxidase on the enzyme-catalyzed ionization of the phenolic group to form a phenolate ion(111).

Morrison *et al.*(112) suggested a mechanism of lactoperoxidase catalysis which involves the oxidation of iodide via a 2-electron transfer to I^+. This reaction could occur in the heme cleft, and I^+ remains within the active center in a nonpolar environment. The tyrosine binding is proximal and I^+ reacts with tyrosine in the nonpolar region to produce MIT. The iodination may be facilitated by virtue of enzyme-mediated base catalysis of phenol to produce the phenolate ion(110).

Marchalonis(113) iodinated immunoglobulins to a specific activity of 5 μCi/μg using carrier-free I-125. There was no evidence of denaturation. The reaction mixture consisted of 250 μg of protein, 1.25μg of lactoperoxidase and 1 μl of I-125 in a total volume of 50 μl. The reaction was started by adding 1 μl of 8.8 mM H_2O_2 and stopped by adding 0.5 ml of 5 mM cysteine or 2-mercaptoethanol.

Miyachi et al.(114) published a series of papers on the iodination of gonadotropins by lactoperoxidase. For Human Chorionic Gonadotropin (HGC), the reaction mixture consisted of 5 μg of HCG, 25 ng of lactoperoxidase and 1 mCi of carrier free I-125 and 25 μl of 0.4 M acetate buffer pH 5.6. The reaction was initiated and maintained by additions of 1-500 ng of hydrogen peroxide at ten second intervals. The biological activities of the preparations were retained and physicochemical analysis of molecular weight and charge suggested that they were undenatured(114).

Thorell and his group(115,116) published a series of papers on the iodination of peptide hormones (FSH, LH, HGH, thyrotropin) to high specific activity (300 μCi/μg) with lactoperoxidase which was isolated from bovine milk. NaI-125 in 8-15 μl (0.5 - 1.8 mCi) was rapidly mixed with 5 μg (25 μl) of peptide at pH 3-8, followed by 4 μg (1.5 μl) of lactoperoxidase and 1 μl of 0.88 mM hydrogen peroxide. After 1-2 seconds the reaction was stopped by adding 500 μl of phosphate buffer. The peptides retained immunological reactivity at I/P ratios of 0.5 to 1.5(115).

Thorell(116) also coupled lactoperoxidase with glutaraldehyde to polyacrylamide, which was used to iodinate proteins, as in the

above procedure, except that 10 µl of polyacrylamide-lactoperoxidase was used. The solid phase was removed from the reaction mixture by centrifugation a 2,000 g for 15 minutes(116).

Hemmaplardh and Morgan(117), iodinated transferrin by the ICl method and by the use of lactoperoxidase insolublized on Enzacryl AA. The insolubilized enzyme (100 mg) was incubated with 5 mg of transferrin 20 µCi of radioiodine, 10 µg of NaI, 0.4 ml of 0.44 mM hydrogen peroxide and 1.6 ml of phosphate buffered saline at pH 7.4 for 15 minutes at 20°C. The enzyme was removed by centrifugation. The in vivo rates of turnover were identical for both methods, as were the rates of uptake by the reticulocytes(117).

Gilbert and Wachsman(118) iodinated bovine fibrinogen using lactoperoxidase insolubilized on Sepharose 6B. Reaction mixtures contained 6.4 mg/ml of fibrinogen, 18.6 µg of lactoperoxidase/ml, 5×10^{-3}M KI, 2 mCi/ml of I-125, 0.15M KCl, and phosphate buffer pH 7.0. Iodination was initiated by adding hydrogen peroxide (final concentration 6×10^{-4}M). Immobilized lactoperoxidase removed by centrifugation(118).

Krohn and Welch(119) used lactoperoxidase to catalyze the iodination of canine fibrinogen. The optimum conditions consisted of 100 µl of pH 8 buffer, 250 µg of protein, 5 µg of enzyme and carrier free iodine; the reactants were incubated at 37°C before adding 10 nM of hydrogen peroxide(119). Efficiency of labelling was 60-85% after 30 minutes. The preparation had a normal molecular weight distribution and isotopic clottability was 83.8%(119). The main derivative was MIT (83.8%). The rate of hydrolysis was 7% in

one day in saline and 1.9% per day in albumin(119). The activity of enzymatically labelled fibrinogen in a surgically induced clot was 66% of the precordial count rate(18). The ratio of thrombus activity to blood activity for enzymatically labelled fibrinogen was 5.1:1 as compared to 8.8:1 for ICl labelled fibrinogen(18).

D. Iodination by Electrochemical Oxidation of Iodide

Rosa and his group(23) developed the electrolytic method of radioiodination of fibrinogen. The method was considered to have the following advantages(23):

1. no oxidizing agent added to the protein solution
2. the rate of formation of iodine could be controlled by controlling the current
3. dilute solutions of carrier iodide could be used to produce specific activities similar to other methods
4. iodination could be carried under physiological conditions.

Rosa et al.(23) used a 20 ml glass beaker as the electrolytic cell. The anode was a 13 cm^2 platinum sheet and the cathode was a platinum wire in a glass tube with the bottom enclosed by a dialysis membrane. The anolyte was 100 mg of fibrinogen in normal saline and 2.5×10^{-4} M KI, all in 10 ml; 0.5 ml of the same KI solution was introduced into the cathode compartment. The yield of labelled fibrinogen was constant from 100-500 uA. Fibrinogen iodinated at a constant current of 300 μCi with a 73% - 77% yield (specific activity 50 μCi/mg) had a turnover time in humans of 5.9 days, which was similar to values obtained for fibrinogen labelled with S-35 by biosynthesis(23).

Rosa(120) also iodinated albumin electrolytically. The 200 mg of albumin was dissolved in 8 ml of saline, to which was added 2 ml of KI-131 or I-125. The pH was 6.5 to 7.0 and the current used was 300 uA. The potential was -490 mV vs SCE. The electrolysis was carried out for 12 hours. Rosa(120) suggested that the iodination occurred on the anodic surface, the iodine reacting with the protein before its diffusion into the solution. It was also noted that the relative percentage of DIT residues increases slowly as the iodine to protein (I/P) ratio increases from 0.2 to 10, and that the relative amount of DIT produced was inversely proportional to the concentration of protein(120). A low iodine discharge rate acted in the same direction. Iodoalbumins at at I/P ratio of 0.5-1.0 were catabolized at a rate similar to the lowest values reported in the literature; at rates greater than 10:1, the catabolic rates were accelerated(120).

The features of the electrolytic radioiodination procedure were(120):

1. iodination occurred at pH 7 with 100% yield without excess iodine (5×10^{-5} M) at 8-10°C
2. the protein was iodinated at a constant rate, the anodic discharge being the rate limiting step
3. the electrolysis procedures were devoid of discernable effects on the protein
4. the process was highly reproducible
5. the inter and intra-molecular distribution of iodine could be controlled.

Teulings and Biggs(121) studied the electrolytic iodination of

fibrinogen(121). They noted that to iodinate the protein at a ratio of 0.5 atoms of iodine per molecule of protein, a concentration of carrier iodide was required which was lower than that used by Rosa's group. This, in turn, produced a high side reaction yield of iodate (121). The electrolytic cell consisted of a 25 ml glass beaker; the anode was a 16.3 cm^2 platinum cylinder and the cathode was a platinum wire in a glass tube. The anolyte was stirred magnetically. The protein was 64 mg of lyophilized fibrinogen in 15 ml of unbuffered saline; 0.1 ml of 10^{-3} M KI was introduced with the desired amount of I-131. The yield was 25% iodofibrinogen, 42% iodate, and 32% iodide after 18 minutes at 300 uA(121).

Teulings and Biggs(121) calculated the anode potentials of several redox equations over a series of oxidation/reduction ratios at pH 6 and pH 7 and iodide concentrations of 6.7 - 20 x 10^{-6} M.

$$2I^- \rightleftharpoons I_2 + 2e^- \qquad -0.536\ V$$

$$H_2O + I^- \rightleftharpoons HIO^- + H^+ + 2e^-$$

$$3H_2O + I^- \rightleftharpoons 6H^+ + IO_3^- + 6e^- \qquad -1.087\ V$$

They also concluded that equation 1 depends on the square of the iodide concentration, but is independent of the pH, and equations 2 and 3 are independent of iodide concentration, but their anode potentials are inversely proportional to pH(121), thus concluding that iodate formation would be eliminated by reducing the pH and increasing the iodide concentration. In the same study, it was also reported that a reduced yield of labelled protein was obtained by decreasing pH. As a consequence, the iodide concentration was increased by a factor of 3 (to 2 x 10^{-5} M), the pH was increased to

pH 8, and the electrolysis time was decreased to 9 minutes(121). The half-life of I-131 labelled protein was 3.6 days which was in agreement with the literature values(121).

Pennisi and Rosa(122) iodinated insulin by constant current electrolysis. In a larger cell, the reaction mixture was 3 ml of 0.086 mM in insulin, 0.15 M in NaCl and 1.2 mM in KI and 100 μCi of I-125. The current was 30 uA for 20 minutes. The yield was 85-90%; specific activity 2-15 mCi/mg; iodine: protein ratio was 0.2. At higher iodination rates, DIT and MIH were formed(122).

The authors found that iodine is incorporated into the insulin at a rate lower than that at which it is formed(122). At I/P ratios of 0.23, 92-98% of the activity was located in iodinated tyrosine. The biological activity of the insulin was not appreciably affected (122).

In another cell, a 2 ml platinum thimble was used as the anode (122). This cell was used for high specific activity iodinations. The protein concentration was 8.6×10^{-6} M, and KI concentration was 1.3×10^{-5} M; the current flow was 6-7 uA for 35-40 minutes. The yield of labelled protein was 80-90%, while the ratio of iodine to protein was 1:1 to 1.2:1 and the specific activity was 150-200 mCi/mg (122). The use of electrolysis for routine preparations was limited to specific activities not greater than 150 mCi/mg(122).

Katz and Bonorris(123) iodinated albumin and amylase electrolytically in a 25 ml platinum crucible. The proteins were dissolved in 0.05 M phosphate buffer pH 7.5 0.1 N in NaCl. A current of 1-2 mA was passed for periods of 30-90 minutes. KI concentration was 10^{-4} M,

albumin concentration was 5.8 x - 6.8 x 10^{-5} M. The yields were 80-90%. The catabolic rate of albumin-fibrinogen was lower than that labelled by the ICl method and the loss of activity of electrolytically labelled amylase was much less than that of the ICl method (123).

Sammon et al.(124) iodinated parathyroid hormone (PTH) electrolytically under controlled electrode potential (rather than constant current) of 0.8 V vs a saturated calomel electrode (SCE). This voltage was sufficient to oxidze iodide to iodine, but not to iodate. The anolyte consisted of 200-400 ug PTH in 1 ml of 25 mM phosphate buffer pH 7.5, containing 0.1 M NaCl as supporting electrolyte, and 21-42 nmole of KI labelled with 2-4 mCi of I-125 was added(124). The cathode was a platinum wire in a glass tube, sealed with dialysis membrane. The electrolysis was carried out at 4°C for 20-30 minutes and gave a yield of 86.5% labelled protein and 13.5% iodate. The preparation was found to be homogeneous by physicochemical techniques and identical to untreated hormone in several bioassay procedures(124).

Caro(125) made a physicochemical study of electrolytic labelling of bovine growth hormone; he used a cell similar to that of Rosa. The anolyte consisted of 30-50 nmole of protein, 200 μCi of I-125, the required amount of KI, made up to 5 ml with Ringer phosphate (pH 7.4) solution. A current of 20 μA was applied for 120 minutes. Relative amounts of MIT and DIT were determined. Only 75% of available tyrosine sites could be iodinated(125).

Donabedian et al.(126) developed micro-electrolytic cell for iodination of polypeptide hormones for radioimmunoassay. The cell

was 5 mm deep by 4 mm in diameter (0.63 ml). The cathode was a platinum wire which acted as a stirrer, while the anode was platinum foil at the base of the cell. The anolyte consisted of 60 µl of saline with 1-5 µg of peptide hormone (insulin, HGH, TSH) and carrier free I-125 to give one iodine atom/molecule of hormone. The iodination was carried out for 45 minutes at 5 µA at room temperature. The yield was 30-50%. The peptides were immunologically reactive in displacing unlabelled hormone(126).

Rollag et al.(127) developed a constant potential thin-layer electrolytic cell for radioiodination of microgram quantities of protein to specfic activities ranging from 200-2,000 Ci/mM. The anolyte consisted of 10 µl of phosphate buffer pH 7.1, 5 µl of protein ($1\text{-}5 \times 10^{-10}$ M) and 1 mCi of I-125 (6×10^{-10} M) in 2 µl. A current was passed at a potential of +0.530 V vs SCE for 10 minutes(127).

Malan et al.(128) electrolytically iodinated glycoprotein and protein hormones. The 30 µl reaction mixtures of 2-10 µg of protein with a 5 molar excess of radioiodide in pH 7.4 phosphate buffer was stirred in a small platinum crucible, and the cathode was a platinum wire and the 2 compartments were connected by a salt bridge through a sintered glass. A constant current of 10 µA was passed for several minutes. In contrast to Rosa, he suggested that the rate limiting step was the incorporation of active iodine into tyrosyl residues. The time for protein iodination was about 20 times greater than the theoretical time for electrolysis of iodide to provide the same level of iodine(128).

He also suggested that the buffer salts have little effect on the electrolysis of iodide. The immunological characteristics of electrolytically labelled hormones compared favourable with chemically iodinated proteins(128).

Fenzi et al.(129) electrolytically iodinated long-acting thyroid stimulator immunoglobulin, and noted a 27% to 77% decrease in biological activity as the I/P ratio was increased from 0.1 to 1.2(129).

Javonovic(130) electrolytically iodinated albumin. The anolyte was composed of 10 ml of 12% of human serum albumin, 10 ml of pyrogen free water, 0.25 ml of 0.05 M KI, 15 drops 1 N NaOH, 50 mCi Na I-131 pH 10. The electrolysis was carried out at a constant current of 1 mA for 4 hours at room temperature. The yield was 90%. The content of labelled amino acid derivatives was determined(130).

Krohn et al.(16) labelled fibrinogen electrolytically by the method of Rosa and of Katz and Bonorris. The anode was a 15ml platinum crucible containing 7 ml of 0.05 M phosphate buffer at pH 7.8, 1-5ml of fibrinogen, carrier free I-125 and 0.1 M in NaCl. The cathode was a platinum wire in a dialysis tube. Reaction times varied from 7-150 minutes, the current from 70-300 μA, and the voltage from 0.6-2.6 volts(16). The yield of labelled fibrinogen was 30-80% (16). The spectroscopic clottability decreased from 97% to 93%(16), the isotopic clottability was 80%(16). The hydrolysis rates were 1.5%/day in plasma, 1.24%/day in albumin, and 27% in one day in saline(16). Gel permeation chromatography showed about 50% aggregation(119). The uptake into surgically induced thrombi produced a

thrombus to precordial ratio of 20:1 compared to 115:1 for ICl labelled fibrinogen(18). A paired comparison of iodination method within a single thrombus showed a thrombus to blood ratio of 8.8:1 for ICl labelled fibrinogen and 1.3:1 for electrolytically labelled fibrinogen(18). They also found that electrolytic labelling oxidized some amino acid residues that were not labelled(19).

Harwig et al.(132) electrolytically iodinated canine fibrinogen, by a modification of the method of Rosa and of Katz and Bonorris. The cell consisted of a 15 ml platinum crucible, and 1 cm platinum wire cathode contained in a dialysis bag. Phosphate, acetate, tris and bartital buffers were tried as electrolytes. The optimum was 0.02 M bartital-0.2 M NaCl pH 7.4. In the other buffers, iodination did not occur at all or occurred only at a slow rate(132). The anolyte was 8 ml of barbital buffer containing 3 mg of fibrinogen, NaI-125, and carrier iodide (1.5×10^{-4} M) to achieve a 150:1 molar ratio of iodide to fibrinogen. The electrolysis was carried out at a +0.4-+0.5V vs SCE(132). The same authors suggest that only the oxidation of iodide to iodine will occur at this potential. At a potential above this range, a considerable amount of fibrinogen precipitated from the anolyte(132). The applied potential ranged from 0.75-1.5 V, and the current from 10-30 μA. The catholyte was 0.5 ml of barbital buffer. The labelling rate was 15-20%/hr and the reaction times were 1.5 to 4.5 hours(132). The optimum pH was 7.4; at lower pH, the labelling rate dropped significantly, and at high pH side reactions could occur(132). The minimum concentration of iodide was 5×10^{-5} M and the minimum concentration of protein was 1×10^{-6} M, below which no labelling occurred(132).

The isotopic clottability varied from 73% for an I/P ratio of 25/1 and 60% for a ratio of 100/1(132). The thrombus to blood ratios were 50:1 and 3:1 for I/P ratios of 25:1 and 100:1, respectively. The half life of the longest component in dogs was 37 hours for an I/P ratio of 25:1, and 10 hours for an I/P ratio of 100:1. The rate of hyrolysis was 2.0%/day(132). At an I/P ratio of 25:1, gel filtration profile was similar to native fibrinogen. At a level of 50:1, a small aggregate peak was observed. At an I/P ratio of 100:1, only 45% of the activity was recovered(132).

Harwig et al.(133) also prepared highly iodinated fibrinogen containing I-123, which was used to image thrombi in animals, as early as 4 hours and as late as 15 hours after induction.

III. METHODS OF LABELLING FIBRINOGEN WITH RADIOISOTOPES OTHER THAN IODIDE

A. In Vitro Methods

1. Technetium-99m

Harwig et al.(139) electrolytically labelled fibrinogen with Tc-99m(139). Two tin wires were inserted through a rubber cap into a 5 ml vial. The reaction mixture was fibrinogen (1 mg/ml), 0.01 M phosphate-0.15 M NaCl buffer pH 6.0, and $Na^{99m}TcO_4$ in a total volume of 2 ml. Current was passed through the cell at 100 μA for 8.3 minutes. The reaction mixture was stirred for 5 minutes after electrolysis. The labelling efficiency was 70-80%. Isotopic clottability was 50-60%(139).

Wong and Mishkin(141) also labelled fibrinogen electrolytically with Tc-99m. HCl (0.05 M) and 2 ml of saline containing 30-60 mCi of $Na^{99m}TcO_4$ were added to a sterile vial equipped with two 0.025 inch diameter zirconium electrodes. A current of 100 mA at 5.5 to 5.7 volts was passed through the solution for 42 to 45 seconds. Fibrinogen solution (0.3 to 0.4 ml containing 4 to 8 mg) was injected slowly into the vial, and incubated for 30 minutes at 37°C. The pH was adjusted to pH 7.0 with serum. The labelling efficiency was 76% and the clottability was 25%(141).

Benjamin(142) described an electrolytic method for labelling albumin which could be adapted to fibrinogen. The procedure entailed the use of a sealed vial with a zirconium needle anode and a zirconium, platinum or BD special ($16x1\frac{5}{16}$) needle as cathode. A mixture of $Na^{99m}TcO_4$ (5 ml), 0.1 ml of 25% HSA and 0.5 ml of 1 N HCl required 4.5 coulombs for adequate radio labelling. After electrolysis, the pH was adjusted with acetate-NaOH buffer or saturated sodium bicarbonate(142).

Harwig et al.(140) studied the *in vivo* behaviour of Tc-99m labelled canine and rabbit fibrinogen. The preparations were stable *in vitro*, but underwent rapid partial exchange of technetium with other plasma proteins and anions *in vivo*, resulting in an early decrease in the amount of Tc-99m associated with fibrinogen. Only 25% of the injected dose remained in the circulation 10 minutes after injection. The half-life of the long component was 25 hours(140). While the denatured fibrinogen was rapidly removed from the circulation, the fraction of clottable fibrinogen remaining was biologically active and was incorporated into thrombi. Higher thrombus to blood

activities were obtained with Tc-99m fibrinogen than with radioiodinated fibrinogen in femoral vein thrombosis in dogs when both agents were injected 4 hours after induction of the thrombosis. Images were obtained of thrombi, beginning 2.5 hours after injection(140).

2. Bromine-77

Knight et al.(145) described a procedure for indirectly labelling fibrinogen with N-succinimidyl-3-(4-hydroxyphenyl propionate) (SHPP) labelled with Br-77. SHPP was labelled by adding 0.1 ml of Na^{77} Br in 0.25 M phosphate buffer pH 2.8, 10 µl of 0.3 mg/ml of chloroperoxidase ,10 µl of dimethylformamide and 15 µl of 0.2 M H_2O_2 to 2 µl of 0.2 mg/ml of SHPP. The labelled SHPP was extracted into benzene and evaporated to dryness before the addition of 4 drops of borate buffer pH 8.5 and 2 mg of fibrinogen(145). After cooling for 15 minutes in ice, fibrinogen was purified by ammonium sulfate precipitation. The labelling efficiencies were about 35-50%. The SHPP to protein ratio was 0.1 to 5(145).

3. Mercury-197

Saha et al.(146) defined the parameters for labelling human fibrinogen with Hg-197. Commercial fibrinogen was dissolved in saline at a concentration 10 mg/ml, to which was added Hg-197 chloride and incubated for 15-30 minutes at 37°C. The pH of the mixture was adjusted by addition of NaOH(146). It was found that the yield of labelling increased to pH 7.0, where it reached a maximum of 90%. However, the material labelled at a pH over 7.0 was insoluble in

phosphate buffer. Repeated precipitation of the preparation with 4 M ammonium sulfate produced a release of 2-5% of the label with each precipitation(146).

4. Indium-111

Goodwin et al.(272) reported the use of In-111 labelled proteins, including fibrinogen. The indium labelling was achieved by means of a bifunctional chelate, utilizing the covalent metal-binding molecule 1-(p-benzenediazonium)-ethylenediamine-N,N,N,N-tetraacetic acid or azophenyl EDTA. The attachment was made between the EDTA - In-111 chelate and the protein through the diazo group. The clottability of the labelled fibrinogen was 53%, with the native fibrinogen having a theoretical clottability of 65%. The distribution and metabolism of bovine fibrinogen labelled with In-111-azophenyl-EDTA was determined in normal and tumor bearing mice.

B. In Vivo Methods

1. Carbon-14

Fibrinogen has been labelled with carbon-14 by several workers, but most effectively by McFarlane(2), who labelled rabbit fibrinogen by administration of C-14 labelled Chlorella protein. Blood samples were removed over a three day period. Radioactivity in the labelled fibrinogen was determined by isolating fibrinogen by ammonium sulfate precipitation, followed by clotting with thrombin. The clot was synerized and combusted before assaying for radioactivity. The biological half-life of the carbon-14 labelled fibrinogen was 69 hours

compared to 66 hours for I-131 labelled fibrinogen(2).

2. Selenium-75

Brodsky et al.(143) labelled fibrinogen and platelets of patients suffering from abnormal coagulation, hepatocellular disease and myeloproliferative diseases by injecting 250 μCi of (Se-75) selenomethionine. Samples were removed at 1,3,5 and 6 hours and daily thereafter for determination of fibrinogen survival(143). The radioactivity in washed clots was determined. Fibrinogen survival time was estimated from the interval between 50% of the maximum radioactivity on the anabolic phase and 50% of the maximum radioactivity on the catabolic phase of the survival curve(143).

Ferguson(144) made a similar study of platelet and fibrinogen survival in normal and diabetic patients. Se-75 selenomethionine (50 μCi) was injected intravenously and blood samples were taken for 18 days(144). Radioactivity in platelets and fibrinogen was assayed and survival times estimated. It was concluded that there was an increased utilization of platelets and fibrinogen in diabetic patients (144).

IV. FIBRINOGEN

A. History of Fibrinogen and Coagulation

The nature of the clotting mechanism has been a subject of observation and speculation dating from the earliest reports of human medicine. Plato wrote "The fibrine is distributed through the blood to secure a proper consistency and prevent it becoming so liquid

owing to heat that it would run away through the porous texture of the body"(148). Aristole compared clotting with the formation of ice from water(149).

The role of fibrinogen was variously attributed to erythrocytes, leucocytes and platelets. The scientific inquiry into the nature of the clotting mechanism began with Malpighi in the 17th century who observed that a white fibrinous substance was obtainable from a red clot by washing with water and that coagulation was delayed by the addition of neutral salts(12).

In the 18th century, Hewson considered that air was the cause of coagulation. He found that some chemicals inhibited coagulation and was thus able to demonstrate that coagulation occurred in the cell-free lymph and not in the formed elements(12). Despite this evidence Home in the early 19th century suggested that erythrocytes consisted of a fibrin nucleus protected by a colored membrance which dissolved and the nuclei aggregated to form a clot(149).

In his classical monograph on coagulation, Virchow (1847)(150) noted that fibrin in putrefying liquids clotted more slowly and this type of fibrin he called fibrinogen. He also believed that the contact of blood with oxygen caused coagulation. This was eventually disproven by Lister(149).

Buchanan(149) first recognized that 'fibrin' pre-existed in the circulation and some agent caused its solidification. In 1859, Denis(151) showed that a clottable substance could be salted out from plasma on saturation with NaCl. He designated this clottable substance as 'plasmine', 'serofibrine' and 'fibrinogen'.

The Swedish biochemist Hammarsten in the late 19th century purified fibrinogen through 3 precipitations with half saturated saline and observed that purified fibrinogen did not clot spontaneously, that clottability decreased with storage and its solubility was dependent on neutral salts(12). He also demonstrated that the formation of fibrin was the specific result of the interaction between thrombin and fibrinogen and demonstrated the role of calcium in the formation of thrombin from prothrombin(12).

About the same time, Schmidt(149) suggested an enzymatic mechanism of coagulation and used such terms as fibrinous substance, fibrin, prothrombin and thrombin. He considered that thrombin acted on two soluble proteins to form soluble fibrin which formed a clot (149).

However, it remained for Morawitz(149) in 1905 to enunciate the classical theory of coagulation by the introduction of the concept of a procoagulation called thromboplastin. Heubner(149) suggested that the hydrolysis of fibrinogen was related to the appearance of fibrin.

During the inter-war period, ammonium sulphate (25-30% saturated) was successfully used to isolate fibrinogen(12). The stability of fibrinogen solutions was improved by adsorption of the prothrombin complex on barium phosphate, calcium phosphate, calcium fluoride, barium sulfate and magnesium hydroxide.

During world war II, Cohen described his landmark procedure of a five variable system for fractionation of plasma proteins in water-ethanol solutions. According to his method 6, Fraction I

contains 40-60% fibrinogen and small quantities of prothrombin and is precipitated from human plasma at 0-3°C in an ethanol concentration of 8%(14). It also contains albumin (7%), alpha-globulin (8%), beta-globulin (15%), and gamma-globulin (9%)(154).

Cohn(152) also reported that under certain conditions, a linear relationship was obtained by plotting the log of solubility against ionic strength, and this relationship was represented by the following equation:

$$\text{Log } S = \beta - K_s \cdot \mu$$

where S is the solubility of the protein, log β is the hypotetical solubility of the protein at ionic strength (μ)=0, and is dependent on pH and temperature, and K_s is the salting out coefficient and is equal to the slope. It is independent of temperature and pH for a given salt and protein. The variation of β with pH reflects the influence of ionization of the protein on solubility in concentrated salt solution. The variation with temperature is dependent on the individual protein(105).

Just after the war, Wäre et al. (153) separated bovine fibrinogen by slowly thawing frozen plasma and washing the undissolved fibrinogen with cold saline. In 1948, Morrison(154) described a procedure for the purification of Cohn fraction I. It was dissolved in citrate buffer pH 6.3, cooled and brought to pH 4.9-5.5 and 0 to 4% ethanol. The Fraction I-A so obtained was 85% clottable, after dissolving in citrate buffer pH 6.3. From Fraction I-A, cold insoluble globulins and occluded proteins were removed at 0°C and 0.5% ethanol. The precipitate was called Fraction I-1 and was 55%

clottable. The supernatant was brought to 8% ethanol and the precipitate obtained was designated as Fraction I-2. This contained fibrinogen in high purity (98% clottable)(154).

Kekwick et al.(155) used 11% diethyl ether by volume at 0°C for precipitation of fibrinogen from plasma. This system was similar to Cohn's procedure except that diethyl ether was used. Further purification of fibrinogen was achieved by washing and extracting at low temperatures with citrate solution, followed by reprecipitation by ether. The preparation was homogeneous on electrophoresis.

In 1956, Blomback and Blomback(12) described what has become a standard method of isolating purified fibrinogen. The procedure begins with Cohn Fraction I of bovine or human origin. This was obtained from blood collected into 1/10 volume of trisodium citrate (3.8%) and centrifuged at 1000 g at 10°C for 60 minutes. Fraction I is obtained by adding ethanol at -3°C to yield a 40-50% clottable fraction. This was extracted twice (E-1 and E-2) with citrate buffer pH 6.0 (0.05% ethanol and 1 M glycine; μ = 0.3, 3°C). Buffer (1000 g/100 g) was added to Fraction I and stirred for 1 hour before centrifuging at 2,000 g for 10-12 minutes at -3°C. The precipitate was Fraction I-0(12). Fraction 1-0 was diluted with citrate buffer 400 ml/100 g and dissolved by warming at +30°C for 30 minutes. The insoluble material was removed by centrifuging at 2,000 g for 10 minutes at room temperature and filtered. It was 87-89% clottable (12).

Fraction I-1 was precipitated from I-0 at a protein concentration of 0.51%, ethanol concentration 2%, glycine concentration 0.12 M,

ionic strength 0.3, and temperature 0°C. The precipitate settled for 30 minutes at 0°C before centrifuging at 0°C and 2000 g. The precipitate contained cold insoluble globulin and fibrinogen; it was 70% clottable(12).

Fraction I-2 was isolated from I-1 at a protein concentration of 0.35%, ethanol concentration of 6.5%, glycine concentration 0.09 M, μ = 0.3, and the temperature was decreased 0° to -4°C. The solution was stirred for 1 hour and the precipitate was collected by centrifugation at 2000 g for 20 minutes at -4°C. Fraction I-2 was 94-97% clottable(12).

Fraction I-3 was obtained from Fraction I-2, which was first dissolved in citrate buffer to a protein concentration of 0.7% and glycine concentration 0.5 M at 0°C (pH 6.4-6.5, μ = 0.1). Fraction I-3 was precipitated at an ethanol concentration of 0.75%, glycine concentration 0.51 molar and protein concentration 0.21%, pH 6.5 and μ = 0.09. It settled for 2 hours at 0°C before centrifuging at 2000 g for 10 minutes at 0°C(12).

Fraction I-4 was obtained from Fraction I-3 at an ethanol concentration of 6.5%, protein concentration of 0.14%, glycine concentration 0.45 M, pH 6.5, μ = 0.08, and the temperature was decreased from 0° to -4°C. The precipitate was centrifuged at 2000 g for 15 minutes at -4°C and dissolved in 0.3 M NaCl-citrate buffer pH 6.3. The clottability was 98-100%(12).

Mosesson and Sherry(156) extended Blomback's work by fractionating Cohn Fraction I into nine fractions. Fractions I-A, I-1, and I-2 were obtained as described previously. Fraction I-5 was obtained

from the supernatant of I-2 by increasing the ethanol concentration to 16% and decreasing the temperature to -4°C. The precipitate was collected by centrifuging at 2,300 g for 20 minutes and contained 5-10% of the plasma fibrinogen and was 91% clottable. Fraction I-6 was obtained from I-5 at 8% ethanol concentration, and Fraction I-7 was obtained from the supernatant of Fraction I-6 at 16% ethanol concentration; they were 92 and 95% clottable respectively. Fraction I-8 was obtained from fraction I-7 by precipitation with 2.1 M glycine at 5°C and centrifuging at 2300 g for 20 minutes; this fraction was 98.7% clottable. Fraction I-9 was obtained from the supernatant of I-8 by ammonium sulfate precipitation at 5°C and centrifuging at 2,300 g for 20 minutes; it was 91.5% clottable(156).

B. Methods of Isolating Plasma Proteins

Plasma proteins can be fractionated from the plasma by methods which differentiate between the molecules on the basis of their physical properties(9,147). The following are some physico-chemical methods of isolating and purifying proteins:

1. molecular size
   - a. gel filtration
   - b. ultra filtration and dialysis
   - c. density gradient centrifugation
   - d. ultracentrifugation

2. solubility
   - a. solvent fractionation
     - i. water soluble organic solvents
   - b. partion chromatography
   - c. salting out and salting in
     - ii. high molecular weight polymers
     - iii. organic cations

iv. small anions
v. polyanions
vi. metallic ions

3. electric charge

a. electrophoretic mobility-electrophoresis
i. starch
ii. polyacrylamide
iii. agarose
iv. moving boundary
v. paper
vi. agar
vii. cellulose acetate
b. isolelectric point manipulation
i. multi-membrance electro-decantation
ii. isolelectric focusing
iii. isotachophoresis
iv. ion exchange chromatography
v. isoelectric precipitation

4. surface properties

a. selective adsorption
b. affinity chromatography
c. immunochemical methods

C. Simplified Procedures for Isolating Fibrinogen

1. Ammonium Sulfate Precipitation:

A popular method of isolation of fibrinogen is that of Laki(157). Freeze-dried Cohn Fraction I is dissolved in 100 ml of 0.1 M phosphate buffer pH 6.4. The solution was diluted with 100 ml of water and refrigerated. Cold insoluble globulin was removed and one-third volume of saturated ammonium sulfate was added to the supernatant. The precipitate was obtained by centrifugation and dissolved in 0.3 M pH 7.4 KCl and dialyzed against KCl solution for three days. Clotta-bility was 95%(157).

Takeda(158) used the following method of isolating fibrinogen for his studies of the metabolism of the protein in humans. Plasma

(20 ml, heparinized) was diluted with an equal volume of 0.09 M sodium citrate and centrifuged for 10 minutes before mixing with a one-third volume of 4 M ammonium sulfate and centrifuged for 5 minutes at 2000 rpm. The precipitate was washed with 1 M ammonium sulfate,dissolved in 15 ml of 0.005 M sodium citrate, reprecipitated and washed, and redissolved in sodium citrate. It was stored at 4°C for 3 hours and centrifuged for 20 minutes(158).

Regoeczi(159) collected 4 volumes of blood into 1 volume of 2% oxalates. Prothrombin was adsorbed on barium sulfate. Fibrinogen of low solubility was precipitated at 18% saturation of ammonium sulfate. The saturation was increased to 23.8% and the precipitate collected by centrifugation at 600 g(159). The pellet was washed with 0.976 M $(NH_4)_2SO_4$ redissolved in 0.9% NaCl containing 0.005 M trisodium citrate. The solution of fibrinogen was dialyzed against the same solvent and precipitation wash resuspension, and dialysis was repeated, before dividing into aliquots and frozen at -25°C. It was 97-98% clottable(159).

McFarlane(2) collected rabbit blood into one-tenth volume of 3.8% sodium citrate and centrifuged for 5 minutes. Plasma was centrifuged at 2000 g for 30 minutes and diluted with 2 volumes of saline. Fibrinogen was precipitated by addition of one volume of saturated ammonium sulfate. After standing for one hour, the precipitate was removed and redissolved in buffer (pH 7.0 phosphate, $\mu = 0.5$) equal to the plasma volume. Fibrinogen was reprecipitated in 23% saturation ammonium sulfate and repeated at 21% saturation, before dissolving in saline citrate buffer pH 6.0. The preparation

was 85 to 95% clottable(2).

Ardaillou(160) removed the prothrombin complex from citrated human plasma by adsorption on 1.75% aluminum hydroxide. Low solubility fibrinogen was eliminated by precipitation with ammonium sulfate at 12% saturation. Fibrinogen was precipitated at 25% saturation, washed, and dissolved in 0.015 M pH 6.0 citrate buffer and the procedure repeated several times before dissolving in 0.3 M NaCl and dialyzed for 14 hours. Cold insoluble precipitate was eliminated by an unspecified procedure(160).

Atenico(16) isolated human or rabbit fibrinogen from citrated, oxalated or heparinized platelet poor plasma by making plasma 25% saturated in ammonium sulfate. After 30 minutes, the precipitate was collected by centrifugation and washed with 25% saturated ammonium sulfate, dissolved in a volume of 0.005 M citrate equal to the plasma volume and reprecipitated, washed, and redissolved in a volume of citrate buffer equal to one-third to one-fifth of the plasma volume. The dissolved fibrinogen was cooled to 3-4°C for 3-12 hours and centrifuged at 0°C for 25 minutes at 4000 rpm to remove cold insoluble globulin. The preparation was 93-95% clottable(161).

2. Glycine Precipitation:

Kazal et al.(13) described a method for isolating fibrinogen from plasma by the use of glycine. Blood was collected into 0.02 ml of 19% sodium citrate and centrifuged twice at 2900 rpm to produce platelet poor plasma. Prothrombin was adsorbed by the addition of 20 mM of magnesium sulfate and 90 g of barium sulfate per liter. The suspension was stirred for 1 hour. The precipitate was removed

by centrifugation and the adsorption was repeated. Glycine (165.15 g/l) was dissolved in the plasma with stirring for 30 minutes and centrifuged for 30 minutes. It was dissolved in a volume of 0.055 M sodium citrate pH 7.4 equal to the barium sulfate supernatant and stirred for 60 minutes before reprecipitation. The fibrinogen was redissolved in one-quarter plasma volume of sodium citrate buffer and was lyophilized in 10 ml aliquots for 48 hours(13).

Silberstein et al.(162) adapted Kazal's method to small amounts of plasma. Sixty ml of blood were collected into 1.2 ml of 19% sodium citrate and centrifuged for 60 minutes at 1800 g. Magnesium sulfate (5 mg/ml of plasma) and barium sulfate (90mg/ml) were added and mixed for 30 minutes to adsorb the prothrombin complex, which was removed by centrifugation at 1800 g for 15 minutes. The adsorption was repeated. Epsilon-amino caproic acid (EACA) (14 mg/ml) was added to the plasma before adding glycine to 65% saturation. The solution was stirred for 30 minutes and centrifuged at 1800 g for 15 minutes. The precipitate was dissolved in 0.055 M sodium citrate pH 7.4 containing 14 mg/ml of EACA. The precipitation procedure was repeated twice(162).

Walker and Catlin(163) described a modification of the method of Kazal. EACA (0.1 M/litre of plasma) and magnesium sulfate (20 mM/l) were added to the plasma and mixed for 10-15 minutes. Barium sulfate (90 g/l) was added and stirred for 1 hour before centrifuging at 2300 g/15 minutes. Triethylaminoethyl cellulose (100 g/l) was added and stirred for 10-30 minutes before centrifuging. EACA (0.1 M/l) and glycine (165.15 g/l) were added and stirred for 30 minutes. The fibrinogen was collected by centrifuging for 15 minutes

and dissolved in 0.055 M sodium citrate pH 7.4 containing 0.1 M EACA. The solution was stirred for 30 minutes and centrifuged for 15 minutes. The fibrinogen was reprecipitated and resuspended in citrate-EACA buffer(163).

3. Beta-Alanine Precipitation:

Straughn and Wagner(164) prepared fibrinogen by adding 6 M beta-alanine to a measured volume of adsorbed plasma to give a final concentration of 1 M. The mixture was placed in an ice bath for 30 minutes and centrifuged for 30 minutes at 2000 g. The supernatant was brought to 2 M beta-alanine. The precipitate was allowed to settle for 30 minutes at 4°C before centrifuging at 9000 g for 20 minutes. The precipitate was dissolved in citrate-saline solvent to the original plasma volume. The fibrinogen was reprecipitated and resuspended in one-third of the original plasma volume of citrate-saline buffer, and clarified by centrifugation(164).

Jakobsen and Kierulf(165) described a method for isolating fibrinogen from 50-100 ml of plasma. Citrated platelet-poor plasma was prepared by collecting blood into one-tenth volume of sodium citrate (4%) and cooling to 4°C. The plasma was removed and EACA was added to a concentration of 0.1 M. Magnesium sulfate was added (0.492 mg/100 ml) and stirred for 15 minutes; 9 g/100 ml of barium sulfate was added and stirred for 60 minutes(165). Adsorbants were removed by centrifugation at 2000 g for 15 minutes and adsorption was repeated. Beta-alanine (6 M) containing 0.1 M EACA was added to the plasma to produce a final concentration of 2.7 M beta alanine and

stirred for 30 minutes, followed by centrifugation at 2000 g for 30 minutes(165). Fibrinogen was dissolved in a volume of veronal buffer (pH 7.35, containing 0.3 M NaCl and 0.1 M EACA) equal to the original plasma volume. The precipitation procedure was repeated twice and the final fibrinogen solution was dissolved in a volume of veronal buffer equal to one-quarter of the original plasma volume. It was then dialyzed against the same buffer for 12 hours. The clottability was 98%(165).

4. Heavy Metal Isolation of Fibrinogen:

Brown and Rothstein(166) used $K_2Hg(SCN)_4$ (potassium tetrathiocyanato-(S) mercurate II)to isolate fibrinogen from human plasma. The blood was collected over ion exchange resin and the prothrombin complex was adsorbed with barium sulfate in the presence of EACA followed by treatment with TEAE cellulose. The pH was adjusted to 7.2 with acetate buffer and made 4 mM in $K_2Hg(SCN)_4$. The precipitate was collected by centrifugation and washed with acetate buffer and dissolved in 0.3 M NaCl and 0.1 M EACA pH 7.2. The $K_2Hg(SCN)_4$ was removed by chromatography on G-25 and adsorption on an ion exchange resin. The product was 94-99% clottable and free of plasminogen, plasmin, and coagulation factors II, V, VIII, X and XIII(166).

5. Cationic Detergent Isolation of Fibrinogen:

Kurioka et al.(167) described a procedure for purifying fibrinogen from ethanol fractionation or Cohn Fraction I. To a 10% solution of Cohn Fraction I in saline containing 1% ethanol at pH 6.3, was added stearyltrimethylammonium chloride. The precipitate

obtained by centrifugation was washed with saline and dissolved in distilled water at 0.015 mM protein and the pH was adjusted to 5.0. The detergent-fibrinogen complex was precipitated by addition of 0.17 M NaCl and resuspended and reprecipitated several times before dissolving in 0.85 M NaCl at pH 8.5. Sodium caprylate was added to a final concentration of 0.03 M. The mixture was centrifuged and ethanol was added to the supernatant to a final concentration of 30%. The clottability was greater than 99%(167).

D. Procedures for Rapid Isolation of Fibrinogen

With the introduction of the fibrinogen uptake test and the noncommitant recognition of the possibility of transfusing serum hepatitis with pooled fibrinogen, there developed a requirement for a method of rapidly isolating fibrinogen from a sample of a patient's own blood. Necessity being the mother of invention, several methods of isolation were developed.

1. Ammonium Sulfate Precipitation:

Frisbie(134) reported a rapid method of isolating and iodinating autologous fibrinogen. Blood was collected into 2 - 10 ml Vacutainers containing EDTA as the anticoagulant and centrifuged at 100C g for 5 minutes. Two 3 ml samples were removed and 9 ml of 30% saturated ammonium sulfate wereadded to each sample, followed by centrifugation at 300 g for 3 minutes. The precipitates were dissolved in 1.5 ml phosphate buffer pH 7.4. The precipitation step was repeated and the precipitate was redissolved in 0.5 ml of buffer.

The total preparation time was 1 hour, including iodination by the chloramine-T method. The product was reported to be 92-97% clottable(134).

Hagen et al.(137) described an isolation and iodination procedure for fibrinogen which could be completed within 1 hour. Twelve ml of blood were centrifuged for 10 minutes and the fibrinogen was precipitated from 4 ml of plasma by adding 2 ml of 3 M ammonium sulfate, followed by centrifugation at 200 g for 5 minutes. The precipitate was dissolved in 4 ml of pH 7.4 0.1 M phosphate buffer containing 0.05 M EACA and 0.38% sodium citrate and reprecipitated. The precipitate was dissolved in 2 ml of buffer. The clottability of the preparation was 85%-90%(137).

Roberts(136) described one of the first rapid methods of isolating fibrinogen. Twenty ml of blood were collected into heparinized tubes and centrifuged at 6000 g for 5 minutes. The plasma was recentrifuged and fibrinogen was precipitated from 4 ml of plasma by addition of 1.3 ml of 4 M ammonium sulfate and centrifugation at 500 g for 5 minutes. The precipitate was dissolved in 4 ml of phosphate buffered saline and reprecipitated and redissolved in 1 ml of buffer(136).

2. Glycine Precipitation:

Hawker and Hawker(135) described a novel method of rapidly isolating human fibrinogen and then iodinating by the chloramine-T method. Fifteen ml of blood were collected into 4.5 ml of 3.8% sodium citrate and centrifuged at 3000 g for 5 minutes; 8 ml of plasma

were stirred with 1 gram of powdered glass and 2 ml of anticoagulant solution containing EACA, zinc sulfate, and sodium heparinate at pH 8.1. Glycine (1.79 M) was added and the mixture was stirred for 8 minutes after which the supernatant was removed by withdrawing it through a sintered plastic disc in the bottom of the stirring vessel which consisted of a modified 20 ml disposable syringe. The precipitate was washed with glycine (1.98 M); 10 ml of anticoagulant solution was added and the precipitation step was repeated and the precipitate was dissolved in 5 ml of saline citrate pH 7.0. It was 97% clottable and the labelling efficiency was 30-60%(135).

Ingraham et al.(168) used glycine in a rapid isolation procedure. They saturated platelet poor plasma to 65-70% and added EACA to inactivate the plasminogen. The product was 92-97% clottable and contained 75-85% of the available fibrinogen with no plasmin present(168).

3. Cryoprecipitation and Polyethylene Glycol Isolation of Fibrinogen

Peabody et al.(138) added 0.1 ml of caprylic alcohol per 100 ml of crushed frozen plasma. Alcohol (53% at -10°C) was added to a concentration of 3%. The slush was warmed to 1°C and centrifuged at 825 g for 30 minutes at 40°C. The cryoprecipitate was dissolved in tris buffer at pH 7.0 and made 0.02 M in sodium citrate and spun to remove the precipitate. Polyethylene glycol 4000 was added to concentration of 8% and the precipitate was centrifuged. The precipitate was dissolved in 0.1 M EACA, 0.2 M glycine pH 6.5 and precipitation

repeated and the fibrinogen was dissolved in 0.13 M NaCl and 0.02 M citrate pH 7.4. The clottability was 93%(138).

E. The Structure and Function of Fibrinogen

1. The Structure of Fibrinogen:

Fibrinogen is one of the largest proteins found in plasma. It is remarkably delicate and is very easy to denature. The commonly accepted molecular weight is 340,000 ± 20,000 daltons(170). Fibrinogen is composed of a twin set of three chains designated as Aa, Bb, and α, with molecular weights of 63,500, 56,000 and 47,000 respectively(170). Fibrinogen thus has the formula $A\alpha_2, B\beta_2, \gamma_2$. Some accepted values of physiochemical parameters of human fibrinogen are given in Table 4(9,10,169,170).

2. Chemical Composition of Fibrinogen:

The amino acid composition of fibrinogen suggests a high surface to volume ratio. The alpha chain has 16 tyrosine residues (β22, γ11) and more arginine and serine than the beta and gamma chains (170); the beta chain has more methionine and cysteine and contains repetitions of Arg-Pro-X and Ala-Pro-X, which is reminiscent of collagen(170). All three chains contain carbohydrate, and none contain free sulfhydryl(170). The number of disulfide bridges reported in the literature range from 21 to 34. There are 19 galactose, 22 mannose, 19 glucosamine and 6 sialic acid groups(170). The carboxy terminal amino acid is valine in all three chains, and

TABLE 4

Physicochemical Parameters of Human Fibrinogen(9,10,169,170)

Physical Constants:

Molecular weight	340,000 daltons
Sedimentation Coefficient($S_{20,w}$)	7.9 S
Translational diffusion coefficient ($D_{20,w}$)	2×10^{-7} cm^2 sec^{-1}
Rotary diffusion coefficient($\theta_{20,w}$)	40,000 sec^{-1}
Intrinsic viscosity	0.25 dl/g
Partial specific volume (v)	0.72
Frictional ratio (f/fo)	2.34
Electrophoretic mobility(u)	2.1×10^{-5} cm^2 sec^{-1} V^{-1}
Isoelectric point (IEP)	5.8
Molecular volume	3.9×10^5 A^3
Extinction Coefficient ($E^{1\%}_{1\ cm}$)	15-16
Alpha-helix (%)	33

Amino Acid Content

	Mole/mole of Protein
Tyrosine	104
Histidine	57
Cysteine	77
Tryptophan	55
Methionine	59
Phenylalanine	95
Serine	230
Total number of amino acids	2896

Fibrinogen Precipitating Solutions

Ethanol	8%
Ammonium Sulfate	0.6 M
Rivanol	0.0065 M
Perchloric acid	0.6 M
Trichloro-acetic acid	0.15 M

alanine, pyrrolidine carboxylic acid, and tyrosine are amino terminal in the alpha, beta, and gamma chains respectively(170). The degree of helicity is about 33%.

3. The Fibrinopeptides:

The fibrinopeptides A and B are split from the alpha and beta chains respectively, by the action of thrombin and a wide variety of serine proteases. They are hydrolyzed at arginine-glycine bonds; A at the 16-17 bond and B at the 14-15 bond; A and B fibrinopeptides thus contain 16 and 14 amino acids respectively(170). In solution, the fibrinopeptides are random coils as determined by circular dichroism(170). In mammals, they have a negative charge of -2 to -6 since B contains tyrosine-O-sulfate,and phosphoserine is present in A(170). Fibrinopeptides are considered able to prevent polymerization of fibrinogen by mutual electrostatic repulsion.

4. Plasmin Degradation of Fibrinogen:

The degradation of fibrinogen by plasmin has been studied in detail by Marder(171). In his terminology, the first intermediate formed is Fragment X with a molecular weight of 240,000 and is still clottable. Fragment X is hydrolyzed by cleavages in the carboxy terminal half of the alpha chain and the removal of 40 amino acids from the amino terminal beta chain. This yields Fragment Y (molecular weight 155,000) and a Fragment D (molecular weight 83,000) both of which inhibit clotting. Fragment Y in turn is hydrolyzed at the amino terminals of all three chains to yield Fragment E, (molecular weight 50,000) which does not inhibit clotting, and another Fragment D.

Fragment E is dimeric and contains the N terminal ends of all three chains, which are held by disulfide bonds(172). It is open and extended. The two D fragments are monomeric and contain segments of all three chains. The D fragments are connected by the three chains to the central fragment E. The carboxy terminals of the alpha chains extend from the D fragments as 'water wings', and thus a major part of the carboxy terminal part of the alpha chain is not connected to the rest of the molecule by disulfide bridges(172).

5. Cyanogen Bromide Degradation:

After degradation of fibrinogen with cyanogen bromide which hydrolyzes the thirty methoionine peptide bonds, Blomback(173) isolated the 'N-terminal disulfide Knot' or N-DSK which contained about half of all the disulfide linkages in the molecule and about 15% of the mass. It was found to correspond approximately to the dimeric Fragment E, in that it contained the amino terminals of all six chains. It also contained the four fibrinopeptides A and B. The primary sequence was determined by Blomback and Blomback(174) and by Takagi and Doolittle(1970). About three hundred of the 1500 amino acids of a half molecule of human fibrinogen have been sequenced, most of these near the amino terminal ends of the three different chains(170).

6. The Conversion of Fibrinogen to Fibrin:

The formation of fibrin fibers is very sensitive to changes in pH, ionic strength, fibrinogen concentration, thrombin concentra-

tion and the presence or absence of some small molecules(170). Fibrinogen can be converted into a gel by:

1. the action of thrombin by the removal of fibrinopeptides A and B,
2. the removal of fibrinopeptide A by reptilase,
3. the neutralization of negative charges by protamine sulfate and
4. the cross-linking of fibrinogen by transamidases(10).

The isoelectric point of fibrin is higher than that of fibrinogen and it has a measurable transverse dipole moment, suggesting the fibrinopeptides are not located symmetrically(170).

The release of fibrinopeptide A occurs much faster than the release of B(10). The removal of the mutually repulsive peptides allows the close approach of the fibrin molecules. They line up with characteristic periodicity as seen in electron microscopy. Polymerization,a highly exothermic reaction, releases 40-50 Kcal/mole, and involves weak interactions including hydrogen bonding, dipole-dipole interactions as well as hydrophobic interactions. Hydrogen bonding is one of the main contributors to polymerization, and to the large negative change in enthalpy(10).

7. Cross-Linking of Fibrin:

After polymerization has occurred, activated factor XIII first cross-links antiparallel fibrin monomers at 2 sites along each carboxy terminal half of the alpha chain. While the gamma chain cross-links are dimeric, the alpha chain cross-links are multi-meric, i.e. several fibrin molecules are cross-linked(10). There may be two

different acceptors in the alpha chains(10). There are no cross-links between beta chains.

8. Paracoagulation:

Paracoagulation can be induced by basic proteins which neutralize the negative charge of fibrinogen, which results in the formation of an ordered fibrinogen polymer with axial perodicity similar to that seen in fibrin(10).

9. Fibrin:

Fibrin appears to be chemically identical with fibrinogen except for the absence of the fibrinopeptides(10). The formation of a fibrin network occurs by, firstly, a linear chain formation depending on the end to end interactions, and secondly, lateral aggregation of the intermediate polymers. It has been suggested that the removal of fibrinopeptide A allows end to end aggregation, while removal of fibrinopeptide B allows side to side aggregation(10).

10. Abnormal Fibrinogens:

There are about 26 reported abnormal fibrinogens, all of which are slow to form clots. They are defective in three ways:

1. abnormal release of fibrinopeptide(s)
2. abnormal polymerization and
3. abnormal stabilization(175).

Fibrinogens Baltimore, Bethesda I, Metz, Giessen, and Cleveland II have been shown to have a defective release of fibrinopeptide A.

Fibrinopeptide A is not released by thrombin from fibrinogen Giessen, and is not released by reptilase from fibrinogen Metz(175). Fibrinogens Detroit and Bethesda I and II are defective in releasing fibrinopeptide B (175). In fibrinogen Detroit, one of the arginine residues in the N-DSK has been replaced by serine, which is repsonsible for the slow polymerization, due to the defective release of fibrinopeptide B. Bethesda I is defective in releasing both fibrinopeptides, but aggregates normally after peptide release. Most dysfibrinogenemias are due to fibrinogens which are defective in polymerization(175). For example, fibrinogens Cleveland, Nancy and Troyes release peptides normally, but aggregate abnormally(175). Fibrinogen Amsterdam aggregates abnormally in the presence of alpha-2-globulin. Fibrinogen Oklahoma is defective in stablization(175).

It has been suggested that abnormal fibrinogens are inherited as autosomal dominants(175). However, normal and abnormal fibrinogens may be present in a single individual as found in fibrinogen Detroit.

11. The Size and Shape of the Fibrinogen Molecule:

The size and shape of the fibrinogen molecule are still under intensive investigation and are a matter of lively debate in the literature. It has long been known from hydrodynamic studies that fibrinogen is either highly asymmetric or highly hydrated. It is a prolate ellipsoid of revolution with an axial ratio of 30 and dimensions of 30 x 90 Å, if it is assumed the molecule is unhydrated (10). The data are also consistent with sphere of 200 Å containing

8 g of water per gram of protein. A compromise structure is a prolate ellipsoid with an axial ratio of 5 and dimensions of 90 x 450 Å and containing a large amount of water(10).

There have been many models proposed as the result of electron microscope studies. Kay and Cuddigan(176) postulated a multinodular molecule 690 Å long. Bang(177) proposed a three nodular model 375 Å long, with an ellipsoid ratio of 6-7. Hall and Slayter(178) obtained electron micrographs of fibrinogen in the form of a linear structure of three nodules held together by a thread. It had the dimensions 475 Å long and diameter of 65 Å for the terminal nodules and 50 Å for the central nodule. The thread-like connecting pieces were postulated to be 8-15 Å thick. The triad morphology accounts for the heavy and light bands observed in fibrin(178).

Koppel(179) produced electron micrographs of bovine fibrinogen indicating a pentagonal dodecahedral structure 240 Å in diameter. The edges of the structure were 81.2 Å long and were to contain the peptide chains. He found periodic structures in fibrin fibers of 200-208 Å and 165-170 Å(179). The volume from these studies was calculated to be 3.4×10^6 Å^3. Each edge of this non-compact form consisted of 2 segments of different peptide chains, which may have the conformation of an alpha helix. Each corner consisted of 3 short parts of different peptide chains. The mass of the protein fibrinogen molecule surrounded ten times more volume of water than its own volume(179).

Hudry-Clergeon *et al.*(180) obtained the same type of results by electron microscopy and suggested the fibrinogen molecule could be

represented by a wire netting sphere with large cavities, freely accessible to the solvent. He suggested that the molecule undergoes a large change in conformation resulting in an unfolding of the molecule in the transition from fibrinogen to fibrin. A periodicity of positive charges (23nm) was seen in fibrin and in paracoagulation but not fibrinogen(180). A conformational change would account for the release of B after the release of A and unmask cross-linking sites on the alpha and gamma chains. Essentially their model consists of a sphere in which the carboxy terminals are folded around the amino terminal knot. It was proposed that this model accounted for many aspects of the physico-chemical properties(180).

The electron micrographs of Pouit et al.(181) showed globular fibrinogen molecules with diameters 190-320 Å and random filamentous extensions. During polymerization, the globules become linear along the filaments and decreased in diameter from 230 Å until they were no longer distinct, while the axial periodicity decreased from 300 Å to 230 Å.

Mosesson(182) suggested that the conclusions of Hall and Slayter were incorrect when they suggested that the monads (single spheres) and dynads (two spheres) were connected into a trinodular molecule since his electrophoretic study showed the electron microscope sample preparation does not cause significant covalent bond disruption.

Blakely et al.(183) in a transmission EM study found globular particles, 10 nm in diameter, singly in triads. They proposed a polymerization process similar to that of Pouit in which swollen spherical structures form fibers by connections of strands of

protein.

Blomback et al.(184) suggested that the N-DSK is situated in the middle of the rod, and the two halves are joined in a parallel fashion, but do not lie in the same plane beyond the linkage region.

The results of Donovan and Mihalyi(185) from differential scanning calorimetry suggest that fragments D and E exist independently as subunits in native fibrinogen in the same conformation in fibrin. Removal of fibrinopeptides does not alter the conformation of stability of the subunits(185).

A characteristic banding, 220-250 Å, is seen in electron micrographs of fibrin after fiber formation. Koppel(179) suggested that it was the diameter of his dodecahedral molecule. Kay and Cuddigan(171) suggested it was a two-thirds over-lap of their 690 Å model. Bang(177) recommended a one-third over-lap of a 375 Å model, while the Hall and Slayter model is consistent with a one-half over-lap of molecules 475 Å long(178).

The transient electric birefrigence studies of Haschemeyer(196) indicated that the fibrinopeptides Å were located at opposite ends of a three ball model fibrinogen, 220 Å from the center, and located on the same side of a transverse axis. The fibrinopeptides B were located equatorially, and fibrin dimers were parallel. On repeating some of this work, Haschemeyer found that the fibrinogen molecule had a nearly spherical structure(186).

Fibrinogen has a swollen or dissociated structure after removal of calcium ions by EDTA(10). Fibrinogen normally has two calicum

ions per molecule. Fibrinogen forms a complex with cobalt II forming a more compact structure and the degree of alpha helicity increases from 23% to 36%(10).

Bachmann et al.(187) investigated the shape of fibrinogen by the freeze-etching technique. The molecules appeared as cylinders with rounded ends 450 Å long and 90 Å in diameter. The volume was 2.9×10^6 Å^3 which was seven times greater than the dry volume, requiring a hydration of 4 grams of water per gram of protein(187).

Lederer and Hammel(188) carried out small angle x-ray scattering measurement of dilute fibrinogen solutions. The results were comptable with a cylinder 450 Å long and 90 Å diameter. Lederer (188) reviewed hydrodynamic studies and suggested that they are compatable only with a cylinder model.

F. Synthesis of Fibrinogen

Fibrinogen is synthesized and stored in the liver(10). The process by which the cell synthesizes three peptide chains and links them by disulfide bonds is unknown. Two methods are theorized(10):

1. one long molecule is synthesized and intra-chain disulfide bonds formed before hydrolysis of peptide bonds to form three separate chains. Hydrolysis might also occur after half molecules are joined;
2. three separate chains are synthesized, assembled into a half molecule, and the half molecules joined by disulfide bonds.

The synthesis rate of fibrinogen appears to be more important

in controlling the plasma fibrinogen concentration than the catabolic rate(189). The factors controlling the anabolic rate are unknown. The normal synthetic rate is 12-20% of capacity, suggesting repression of genes controlling synthesis. The synthetic rate does not appear to be controlled by fibrinogen plasma concentration. The factor(s) causing an increase in acute phase reactants (fibrinogen, ceruloplasmin, haptoglobulin, and α-1- antitrypsin) after a pathological stimulus are also unknown(189).

Regoeczi(189) calculated the synthetic activity of hepatocytes from the catabolic rates, assuming a steady state condition. A synthetic rate of 34 mg/kg/day amounts to 2.4 grams or 4.22×10^{18} molecules synthesized per day in a standard man. A liver parenchymal cell must synthesize 23.5 million molecules per day or 271 per second. If there are approximately 3000 amino acids per molecule, the cell will assemble 785,000 amino acids per second into fibringoen molecules.

G. Catabolism of Fibrinogen

The fundamental metabolic characteristics of fibrinogen include a high metabolic rate, a small extravascular compartment and a first order diffusion and catabolic rates(189). The catabolic rate can be determined by either measuring the catabolized urinary activity and protein bound plasma activity over the same time period, or by measuring the plasma activity alone. Regeoeczi(189) suggests that the former method is not useful for fibrinogen because if the half-life is short, the production of catabolites exceeds the elimination rate and so the urinary radioactivity is not in equilib-

rium with the plasma activity of metabolites. A discussion of compartmental models is outside the scope of this survey.

Fibrinogen catabolic rates are consistent with a catabolism of plasma fibrinogen by pinocytosis, since sudden changes in fibrinogen pool size lead to rapid changes in the catabolic rate (189). Catabolism may occur in the endothelium or pericapillary histiocytes or the reticuloendothelial system(190). It is generally accepted that fibrinogen is not converted to fibrin before catabolism.

The catabolism of fibrinogen is a first order reaction. When the logarithm of decreasing plasma activity of labelled fibrinogen is plotted against time, and terminal linear portion is extrapolated to zero time, the intercept is C_1 and the slope of the line $-a_1$. If the ordinate values of this line are subtracted from the plasma curve values, a new curve is obtained, which, when plotted on semi-log paper yields another straight portion with intercept C_2 and decay constant a_2. This process may be repeated to yield intercept C_3 and decay constant a_3. Thus $C_1+C_2+C_3=1$.

Since the rate of disappearance of fibrinogen is generally a double exponential function, the rate of disappearance is:

$$F = C_1e^{-a_1t} + C_2e^{-a_2t}$$ where F is the concentration of plasma fibrinogen and a is the fraction of fibrinogen catabolized per unit time and equals $\ln 2/t_{\frac{1}{2}}$.

Matthew(191) developmed a multi-compartmental model for plasma protein catabolism. Using the compartmental model and the catabolic parameters C_1, a_1, C_2 and a_2, the fractional catabolic rate is $k_m = (C_1/a_1 + C_2/a_2)^{-1}$. This is the reciprocal of the area under

the plasma clearance curve. The rates of protein transfer to and from the extravascular compartment are, respectively(189),

$$k_{1,2} = C_1C_2(a_2 - a_1)^2/(C_1a_2 + C_2a_1) = C_1b_1 + C_2b_2 - k_m$$

$$k_{2,1} = C_1a_2 + C_2a_1 = a_1 + a_2 - (k_m + k_{1,2})$$

The total body fibrinogen is given by(189)

$$F_t = (C_1/a_1^2 + C_2/a_2^2)x\ (C_1/a_1 + C_2/a2)^{-2}$$

and the extravascular fibrinogen is then $F_t - 1$. The absolute catabolic rate is calculated from the fibrinogen concentration, x the intravascular volume x fractional catabolic rate divided by the body weight. The ratio of the extravascular compartment (ev) to the intravascular compartment (iv) is given by

$$ev/iv = (C_2(a_2 - a_1)\ /(a_1C_2 + a_2C_1)$$

and the intravascular compartment fraction is calculated by

$$iv = (k_{1,2}/k_{2,1} + 1)^{-1} \text{ and } ev = k_{1,2}/k_{2,1}$$

A summary of fibrinogen metabolic parameters is given in Table 5.

H. Radio-Labelled Fibrinogen Studies

Ardaillou and Larrieu(210) studied fibrinogen radioiodinated by the iodine monochloride (ICl), lactoperoxidase (LP) and thyroid peroxidase (TP) methods. The yield by the ICl method was 50%, while the LP produced a yield of 90%, and the TP 80%. The isotopic clottability was 75% by the TP method and 85% for the other methods(210). Approximately 95% of radioactivity was found in the fibrinogen peak of the ICl and LP methods, but only 86% of the TP method when the preparations were chromatographed on Sepharose 4B(210). Less than

TABLE 5(1)
Metabolic Parameters of Some Mammalian Fibrinogen

Author	Year	Species	$T_{\frac{1}{2}}$ hr	C_X	FCR d^{-1}	ACR mg/kg/d	iv	$k_{1,3}$ d^{-1}	$k_{3,1}$ d^{-1}
Amris(205)	1964	man	103.2						
Ardaillou(160)	1974	man	92.4		0.114		0.787	0.366	5.48
Boneu(192)	1976	man	93.4						
Bradley(194)	1975	man	100.2	0.64	0.25				
Brodsky(143)	1970	man	156						
Caretta(203)	1977	man	56						
Charkes(92)	1974	man	122.4						
Collen(196)	1971	man	99.4 11.5	0.67	0.24	28	0.72	0.60	1.02
Covey(204)	1975	man	96						
Davies(197)	1973	man	99.4						
Ferguson(144)	1974	man	165.6						
Hickman(195)	1971	man	96	0.77	0.225				
McFarlane(198)	1964	man	69.8		0.31	41.3	0.773		
Regoeczi(189)	1974	man	93.6		0.23	34	0.81	0.453	1.93
Rosa(23)	1964	man	142						
Teulings(121)	1970	man	86.4						
van der Maas(193)	1971	man	91.2						
Zetterqvist(202)	1969	man	109.2						

TABLE 5(2)

Metabolic Parameters of Some Mammalian Fibrinogens

Author	Year	Species	$T_{\frac{1}{2}}$ hr	C_x	FCR d^{-1}	ACR mg/kg/d	iv	$k_{1,3}$ d^{-1}	$k_{3,1}$ d^{-1}
Cohen(200)	1956	rabbit	66						
Colombetti(207)	1976	dog	36.0						
Dugan(206)	1973	dog	2.2 103.2	0.5 0.5	0.0132				
Hagan(137)	1974	dog	55	0.45					
Harwig(132)	1975	dog	52						
Lazewatsky(93)	1976	cow	122.4						
Mahn(209)	1975	rabbit	40.6		0.598	58.9	mg/kg/m^2		
McFarlane(2)	1963	rabbit	61 6	0.8 0.2	0.333				
Metzger(131)	1973	dog	0.64 4.73 40.5	0.26 0.391 0.343	1.043				
Owen(199)	1974	dog	119.4						
Regoeczi(159)	1970	rabbit	120		0.39	37.4		0.45	
Takeda(208)	1972	cow	74.4						
Tytgat(201)	1971	dog	60.9	0.65	0.39	58		0.79	1.36
Zetterqvist(202)	1969	dog	48						

2% remained on the top of the column of the former two preparations and less than 5% of the latter. No activity was obtained in the void volume and the elution profiles were similar to those of unlabelled fibrinogen. Polyacrylamide gel electrophoresis patterns were similar for native and labelled fibrinogens. The hydrolysis rate in saline was 10% after 4 days and less than 5% in plasma(210). LP and ICl preparations gave similar results to native fibrinogen on polymerization, whereas TP sometimes gave variable results(210).

Ly and Kierulf(211) studied the effects of increasing iodination on the in vitro properties of human fibrinogen. When the iodine to protein ratio exceeded three, aggregation and shortening of the thrombin clotting time were observed. Production of N-terminal glycine was similar in native and iodinated fibrinogen. At visible gelation, heavily iodinated fibrinogen (iodine to protein ratio = 20) had enhanced fibrin polymerization. Extensive iodination also produced increased electrophoretic mobility. This was due to the increased negative charge caused by dissociation of the phenyl hydroxyl of the MIT and DIT. Clottability was unchanged at I/P ratios from 0.4 to 20(211).

Regoeczi(213) measured the change in clottability of fibrinogen labelled with I-125 with I/P ratios ranging from 0.06 to 9.96 in the case of human fibrinogen, 0.11 to 5.37 for rabbit fibrinogen, and 0.01 to 4.28 in the sheep fibrinogen. No substitution dependent changes in clottabilities were observed, but Regoeczi suggested that heavily iodinated fibrinogen clots more slowly(213).

Regoeczi(212) also reviewed the phenomenon of double half-life

fibrinogen, in which the mixing period is followed by two consecutive exponentials. This process could be due to the presence of a substance which stimulates the RES, or a portion of the preparation may have a higher affinity for the catabolic cells(212). This occurs frequently in old or improperly stored fibrinogen preparations(212).

Krohn et al.(16) studied the properties of fibrinogen labelled by the iodine monochloride, lactoperoxidase, chloramine-T and electrochemical methods. He found that in the case of the chloramine-T preparation only 10% of the product corresponded in molecular weight to native fibrinogen(16). Most of the preparation (80%) was in the form of aggregates(16). These aggregates have been shown by Metzger to accumulate in the liver when injected intravenously in dogs(131). Krohn found that 47% of the electrolytic preparation was always found in the form of aggregates(16). He also observed that the ICl and enzymatic preparations were considerably more stable to hydrolysis than the other preparations(16).

Metzger(17) studied the clearance of fibrinogen labelled by these four methods and found that the fraction of fibrinogen eliminated with the half life corresponding to native fibrinogen decreased in the order ICl, enzymatic, electrolytic and chloramine-T(17). The first two methods had approximately twice the activity clearing with the longest half life(17).

Coleman et al.(18) studied the clot to blood ratios in surgically induced clots. He found that the clot to blood ratios of activity were approximately 9,6,2.5, and 1.5 for the ICl, lactoperoxidase, chloramine-T, and electrolytic methods respectively(18).

Harwig et al.(214) measured the effect of iodination level on the properties of radioiodinated canine, rabbit and human fibrinogen. Iodination with 3 to 9 iodine atoms per molecule resulted in unchanged isotopic clottability compared to preparations labelled at an I/P ratio of 0.5(214). No alteration in the molecular weight was observed by sodium dodecylsulfate (SDS) gel electrophoresis. Canine and human fibrinogen labelled up to I/P ratios of 4.5 demonstrated little change in biological clearance rate (70 hours and 78 hours respectively). Rabbit fibrinogen iodinated with 3.5 atoms of iodine per molecule was eliminated more rapidly than at an I/P ratio of 0.5 (52 hours versus 47 hours)(214).

I. The Clinical Use of Radiolabelled Fibrinogen

Labelled fibrinogen has been used in medicine in two ways. It can be injected intravenously where it behaves in a fashion similar to native fibrinogen, in that it participates in any thrombus or fibrin formation. The labelled fibrinogen can therefore be monitored externally at any site of interest to detect the presence of accumulating fibrin. This ability has been used for the detection of post-operative deep venous thrombosis of the lower limbs, pulmonary emboli, pulmonary trapping of fibrin in microembolism syndrome, coronary emboli, and renal transplant rejection. Labelled fibrinogen can also be used to study the catabolic rate of fibrinogen by serial blood sampling in patients suffering from a variety of pathological conditions.

1. External Monitoring of Labelled Fibrinogen:

McFarlane(2) demonstrated that labelled fibrinogen is metabolized at a similar rate to that of native fibrinogen. The first preparations of labelled fibrinogen for human use were made by Christensen(218) in 1958 and by Hammond and Verel(219) in 1959.

a. Detection of Deep Venous Thrombosis:

Hobbs and Davies(3) first showed experimentally that a forming thrombus incorporated I-131 labelled fibrinogen that could be detected by external counting over a limb. Palko et al.(4) used this technique clinically to locate deep venous thrombosis. However, it was Atkins and Hawkins(5) who successfully used I-125 labelled fibrinogen in patients with deep venous thrombosis.

The method was refined by British surgeons such as Flanc et al. (6), and Negus et al.(7) who confirmed the reliability of the fibrinogen uptake test (FUT) by comparing it with phlebography. They showed that if the labelled fibrinogen was injected into surgical patients before thrombus formation, they were able to detect 90% of the deep vein thrombi, that could be found with phlebography. The procedure consists of marking points along the leg from the center of the groin along the course of the femoral vein in the thigh and then down the posteriormedial aspect of the calf. The detector consists of a NaI crystal scintillation system, the output of which is fed to a scaler and timer or to a rate meter. Activity is measured on the skin at marked points and by pointing the crystal towards the femoral

vein in the thigh and into the bulk of the calf muscle in the leg better resolution is obtained. The FUT is an accurate method of diagnosis of deep venous thrombosis (DVT) in the lower two-thirds of the thigh and in the calf. The presence of a thrombus is suspected if a count rate at a point on the leg expressed as a percent of the precordial count is 15-20% higher than adjacent points or the same point on the opposite leg or is higher over a 48 hour period.

Kakkar et al.(8) simplified the procedure by the use of a rate meter attached to a gamma detector. In his procedure the crystal probe is placed over the 4th left intercostal space and the radioactivity over the heart is measured and the machine adjusted to represent a reading of 100%. All other readings are obtained as a percentage of the precordial count. An increase of 20% in any value represents the formation of a thrombus.

Kakkar et al.(216) studied the natural history of DVT in 132 surgical patients by the use of 100 μCi of I-125 fibrinogen. They concluded that most thromboses begin in the tibial and soleal veins and propagate to the popliteal and femoral veins. The FUT could be used to detect patients at risk of pulmonary embolism(216).

Flanc et al.(6) used the same technique in post-operative patients. They found the calf to be the most common site of thrombosis and suggested the FUT to be the most sensitive method of detection of DVT(6).

Warlow(217) used the FUT to measure the incidence of DVT in stroke patients suffering paralysis in one leg. Over half had DVT

in the paralyzed leg within 10 days(217).

Hume et al.(220) performed I-125 fibrinogen leg scanning in 157 patients who had undergone hip replacement. Warfarin, sudoxicam, heparin and placebo were compared for prophylactic effect. Fifty-five positive results were detected(220).

Hicks and Hazell(222) determined the risk of hepatitis after the use of I-125 fibrinogen in 354 post-operative patients. Fibrinogen from two sources was used to measure DVT in a clinical trial of aspirin. There were 4 deaths in the controls and 3 in the fibrinogen group due to jaundice. Laboratory studies of 252 patients indicated no excess of subclinical liver disease.

Harris et al.(221) carried out flat field probe scanning with I-125 fibrinogen as a tracer for DVT after hip replacement in 83 patients. Accuracy of the technique was 76% or 83% excluding errors due to the wound;the sensitivity was 49%(221).

There are a number of disadvantages to the fibrinogen uptake test(215):

1. It cannot detect thrombi above the level of the upper third of the thigh.
2. It is necessary to block the uptake of radioiodine by administration of stable iodine.
3. It is necessary to wait 24 hours after administration of the radiopharmaceutical before testing the legs.
4. Hepatitis virus may be present in non-autologous preparations.

The advantages of the test include(215):

1. It is the best method for screening large numbers of patients for DVT in clinical trials of prophylactic drugs and physical therapy.
2. It is useful for screening high risk patients.
3. It can be used to make a diagnosis in cases of suspected established DVT.
4. It is useful for following the natural history of DVT in hospitalized patients.

The discussion of the physical methods of diagnosis is outside the scope of this survey. However, Johnson reviewed the accuracy (total correct diagnosis/total number examined)(223), the specificity (number of normal limbs diagnosed/total number of normal limbs), and the sensitivity (number of thrombosis diagnosed/total number of thrombosis), as compared to contrast phlebography(223).

Method	Accuracy(%)	Sensitivity(%)	Specificity(%)
Clinical	54.9	57.7	52.2
Plethysmographic	75.2	68.2	82.1
Doppler	76.8	75.6	78.0
Fibrinogen (prospective)	95.5	93.0	98.0
Fibrinogen (diagnostic)	61.5	45.0	78.0

Charkes et al.(92) have suggested that since I-125 fibrinogen cannot be used to detect DVT in the iliac veins, I-131 labelled fibrinogen should be used to locate DVT in these veins. This radiopharmaceutical was used in 37 patients. Whole body scans were

performed up to seven days after injection of 350 μCi of I-131 fibrinogen. Compared to phlebography, the method was 93% accurate; the sensitivity was 70% and the specificity was 95%(92).

b. Diagnosis of Deep Venous Thrombosis by Isotopic Methods:

Rhodes et al.(224) have divided the thrombosis detecting radiopharmaceuticals into six categories, which correspond to pathways of incorporation during thrombus formation and dissolution:

	Stage	Tracer
1.	Thrombogenesis	Platelets Clotting Factors Fibrinogen
2.	Clot Stabilization	Factor XIII
3.	Adhesion or adsorption	Platelets Macro-aggregated Albumin RBC Microspheres Sodium Pertechnetate
4.	Invasion	Leucocytes
5.	Antigen-antibody reaction	Anti-fibrin Anti-fibrinogen Anti-plasmin Anti-Factor XIII
6.	Fibrinolysis	Plasmin Plasminogen Streptokinase Urokinase

Duffy et al.(225) described the use of macro-aggregated albumin labelled with I-131. After injection of 100 μCi of the tracer in the pedal vein activity was measured at seven points along the injected leg and the control leg. The clearance of activity was either rapid or delayed. The sensitivity was 88%, specificity 87% and the accuracy

87%.

Freeman et al.(226) reported a single case in which Tc-99m sulfur colloid was useful in inadvertently locating a very large thrombus in the superior vena cava, which was caused by the presence of a catheter.

Webber et al.(227) injected Tc-99m MAA into pedal veins of patients and scanning of the lower extremities showed a 70% accuracy compared with venography. Of patients with pulmonary emboli, 80% had positive leg scans. There was a high incidence of false positives(227).

Henkin and Quinn(223) studied human albumin microspheres as a tracer for DVT and found a 96% accuracy compared with contrast venography(228). The calf could not be diagnosed accurately. False positives were almost absent. They considered macro-aggregated albumin inferior to human albumin microspheres. The ability to image the lungs provided a check on the patient with venous disease or a check on the progress of previous emboli(228).

Kempi and von Scheele(229) used $Na^{99m}TcO_4$ to image thrombi in the deep venous system of the legs. The tracer was injected in the brachial vein and scintigrams of the legs were taken every 20 seconds for 3 minutes. Four hours later, a single image of the legs was obtained. The ratio of radioactivity in an area of the suspected thrombi was compared with a similar area on the opposite leg. A ratio greater than 1.15 was considered positive. The accuracy was 80%, the sensitivity was 90%, and specificity was 67%.

Kwaan and Grumet(230) reported the use of Cr-51 labelled

leucocytes for detecting preformed thrombi by external scintillation counting. Labelled leucocytes infiltrated throughout the thrombus. The uptake of labelled autologous leukocytes in patients with clinically evident DVT correlated well with venographic diagnosis. An 8-20% increase in radioactivity occurred at 12-24 hours.

Charkes et al. (240) found that I-131 labelled leucocytes rapidly accumulated in venous thrombi in dogs. Tc-99m sulfur colloid ingested by leucocytes was considered more successful. Thrombi could be detected after labelling by this method by photoscanning in dogs, with thrombus to blood ratios of 50:1 at 24 hours(240).

Thakur et al.(241) labelled platelets with Indium-111-8-hydroxyquinoline complex with 95% efficiency. The thrombi were detected in dog veins by imaging 3 hours after administration. The thrombus to blood ratio was 15:1. Damaged carotid arteries demonstrated thrombus to blood ratios between 8 to 20:(241).

Reich et al.(231) described the use of I-131 horse antihuman fibrin-fibrinogen globulin to label asymptomatic or symptomatic calf vein thrombus(231). The use of a scintillation detector permitted the localization of symptomatic calf thrombus and asymtomatic thrombus was diagnosed 3 days before clinical diagnosis(231).

Spar et al.(232) located thrombi in dogs with I-131 anti-canine fibrinogen. In humans, it was found to have a long half-life. Circulating labelled antibody could be removed by immunological means(232).

Bosnjakovic et al.(233) labelled rabbit anti-human fibrin globulin with I-131 by an electrolytic method. Administration to patients allowed detection of forming and formed thrombi and could

discriminate between acute thrombosis and chronic varicosities(233).

Harwig et al.(234)isolated canine plasminogen by affinity chromatography on lysine Sepharose 4B and labelled it with I-125 or I-131 by the ICl method. Radioiodinated plasminogen produced thrombus to blood ratios of 7.8:1 in dogs with 2 day old thrombi. Thrombi six days old could be visualized 80% of the time(234).

Millar and Smith(235) labelled urokinase with Tc-99m by a stannous chloride method. Twenty minutes after the injection of 3 mCi of the tracer, scanning was started. The accuracy was 95.5% the sensitivity was 91.6% and the specificity was 100%. The method detected formed and forming thrombi, was completed within 1 hour, and gave visualization of the calf, femoral and ileofemoral veins (235).

Rhodes et al.(236) labelled urokinase with radioiodine, and with Tc-99m. They found that urokinase retained its enzymatic activity and immunoreactivity over a wide pH range. In vivo it concentrated in the liver and kidneys and was excreted in the urine. It localized in thrombi induced in the veins of dogs(236).

Dugan et al.(237) located surgically induced thrombi in dogs with Tc-99m labelled streptokinase. The enzyme was labelled by use of a $SnCl_2$-HCl procedure. The tracer was eliminated with a half-life of 12 minutes, while the remainder had an 85 minute half-life. Ten minutes after injecting 3 mCi of the tracer, positive uptake was noted; the optimum occurred at 1 hour. The ratios or radioactivity in the clot to blood was greater than 20:1 at 4 hours and 2.3% of the injected dose was incorporated(237).

Kempi et al.(238) labelled streptokinase with Tc-99m. The pH of the preparation was 2.0 and 50% of the pertechnetate was not bound to the streptokinase. A gamma camera was used to image both legs. A ratio of 1.05:1 at the suspected site versus a corresponding site in the opposite leg was diagnosed as pathological. In 19 patients, the accuracy was 89.5%, compared to venography, the sensitivity was 84.6%, and the specificity was 100%(235).

Siegel et al.(239) labelled streptokinase with I-131 by the Chloramine-T method and found that it localized in induced clots in dogs, permitting the imaging of peripheral and pulmonary emboli.

Rhodes et al.(224) labelled highly purified streptokinase with I-131 and found that the tracer localized in experimental clots in dogs with clot to blood ratios from 1.8 to 3.3:1 within 2 hours. The blood clearance rate was slower and the distribution was different than reported for previous preparations(224).

c. Pulmonary Embolism:

While I-125 fibrinogen cannot be used for imaging thrombi in the chest, Busch et al.(242) used the radiopharmaceutical to detect fibrin trapping in the lungs of multiply injured patients developing the microembolism syndrome. Uptake was detected in four patients out of a total of 15, which was confirmed at autopsy in these four(242).

d. Intracardiac Thrombosis:

Frisbie et al.(243) used I-131 fibrinogen to survey 20 patients

with cardiac disease predisposing to intracardiac thrombosis: 8 of 9 patients with positive uptake and 11 of 11 patients with no uptake were confirmed at surgery or autopsy. The method of detection was conducted by imaging or rectilinear scan at 5 minutes, 3 hours, 24 hours, and from 2 to 6 days after injection of the tracer(243).

Erhardt and Sjogren(244) administered 100 μCi of I-125 fibrinogen to 80 patients in an attempt to diagnose ventricular mural thrombi complicating myocardial infraction. Precordial activity was measured at 4 sites for 6 days. A sustained rise in activity was suggestive of mural thrombosis. Using this technique Erhardt concluded that in acute myocardial infarction, coronary thrombosis formation may be secondary and takes place over a long time period(244).

Warlow and Terry(245) observed that 4 of 83 patients with acute myocardial infarction had no decreased precordial activity after injection of 100 μCi of I-125 fibrinogen. A sustained or rising precordial activity can detect intracardiac mural thrombosis before embolization(245).

e. Detection of Renal Transplant Rejection:

In 1967, Porter(246) observed intravascular fibrin in rejected renal transplants. Salaman(247) published a series of papers on the use of I-125 fibrinogen for the detection of rejection of cadaveric renal transplants. After administration of the labelled protein, the radioactivity of the transplant was measured with a scintillation

counter(247). An increase of 20% over the precordial count rate was found in transplants undergoing rejection. In a study of rejected kidneys, he found fibrin in the renal interstitium and within the glomeruli. Of 11 patients, 7 showed increased activity and all had other evidence of rejection. It was suggested that the procedure was valuable in patients with delayed function and those with suspected rejection diagnosed by other methods(247).

Straub(248) also investigated the method and suggested the procedure was only valid after healing had occurred. Other errors in the procedure were the formation of hematomas, full bladders, and ureteral obstruction. Fibrinogen turnover studies could not be used to detect transplant rejection. However, the method could be used to evaluate heparin therapy(248).

Winston et al.(249) used I-131 fibrinogen as an indicator of renal rejection by means of a scinitllation camera. Transplant uptake levels of less than 0.3% of the injected dose indicated non-rejection; levels from 0.3 to 1.0% of the injected dose were of 'intermediate significance', requiring follow up. Accumulation of more than 1% of the dose occurred during or preceeding rejection episodes(249).

George et al.(250) made an evaluation of renal transplant rejection with labelled fibrinogen, ^{99m}Tc sulfur colloid and ^{67}Ga-citrate. It was concluded that ^{99m}Tc sulfur colloid was superior to radiofibrinogen and gallium in imaging qualities, radiation dose, sensitivity and specificity(250).

f. Detection of the Uptake of Labelled Fibrinogen by Tumors:

Monasterio et al.(251)used I-131 and I-125 labelled fibrinogen to detect malignant tumors in 96 patients. Electrolytically labelled fibrinogen was administered to the patients with space occupying lesions, including inflammations and cysts. Activity in the lesion was expressed as a percent of the precordial count. Correct diagnosis was obtained in 75% of the cases(251).

Riccioni used I-131 fibrinogen to evaluate cold spots of liver radio-colloid scans(252). Activity of I-131 fibrinogen in these areas were expressed as percent of the precordial count rate at 1 and 48 hours. The concordance was 87%, the specificity 91%, and the sensitivity was 84.2%(252).

Krohn et al.(253) found I-123 fibrinogen had a higher uptake in Murine tumors than ^{67}Ga-citrate, ^{111}In-bleomycin, or Iodo-bleomycin. The I/P ratio was 50:1(253).

De Nardo et al.(254) labelled fibrinogen at I/P ratios of 2,5,25,45 and 65 to 1. The distribution and clearance was measured in the KHJJ tumor model. Blood clearance of heavily iodinated fibrinogen was much faster than fibrinogen with an I/P of 2 and the tumor uptake was seven times greater at 4 hours(254).

2. The Measurement of Fibrinogen Catabolic Rates in Various Disease States

a. Renal Disease:

Ardaillou et al.(160) studied the kinetics of I-125 fibrinogen

in patients with chronic renal failure of arterial origin and of other causes and in control patients. It was concluded that synthesis of fibrinogen in chronic renal failure was increased in all cases(160).

A study of fibrinogen catabolism by Wardle and Kerr in patients with glomerulonephritis demonstrated increased fibrinogen catabolism due to active immune complex disorders(255).

b. Cancer:

Bettigole et al.(256) measured the half-lives of labelled fibrinogen in patients with various malignancies. They ranged from 30 to 86 hours. Patients with intravascular coagulation syndromes had fibrinogen half-lives of 10-16 hours.

Boneu et al.(192) found that the half-life of fibrinogen I-131 was shortened in patients suffering from malignant tumors (2.5 days versus 3.9 days for controls). External counting procedures demonstrated the uptake of labelled fibrinogen in the tumors. Administration of heparin restored the half-life to normal values (192).

McFarlane et al.(198) investigated the metabolism of fibrinogen in patients suffering from primary carcinoma of the liver, cirrhosis of the liver, and acute pulmonary tuberculosis. He found that neither the intravenous fraction nor the fractional catabolic rate changed but the total mass of fibrinogen catabolized was increased, the change being greatest in the tuberculosis (198).

Laki(257) observed that when the clot stabilization process was inhibited in mice, implanted YPC-1 tumors did not take or were retarded in growth. This was considered to be due to the prevention of the formation of the matrix into which new capillaries grow into the malignant tumors, forcing the cells into a vegetative existance. Ogura et al.(258) studied the localization of I-131 fibrinogen in Walker carcinosarcoma of rats(258). Fibrinogen localized in the tumor in a large quanitity and with high specificity, which was closely related to tumor growth or development after implantation. The degree of localization of fibrinogen was proportional to original clottability of the preparation. It was suggested that fibrinogen localized in the tumor tissue as fibrin(258).

Peterson and Appelgren(259) measured the uptake and retention of labelled albumin and fibrinogen in a transplantable rat tumor. A high tumor uptake of both proteins was noted and that was considered to be due to high permeability of the tumor capillary walls(259).

c. Other Disease States:

Tytgat et al.(260) studied the fibrinogen turnover in cases of polycythemia, thrombocytosis, hemophilia A, congenital afibrinogenemia, and during streptokinase therapy. The fibrinogen half-life in control patients was found to be 4.14 days. In primary or secondary polycythemia, the fibrinogen half-life was shorted to 2.9 days and in patients with thrombocytosis, the half-life was 3.2 days; in hemophilia A, the half-life was 3.7 days. The half-life was prolonged in all conditions, except hemophilia, by anticoagulation. Fibrinogen turn-

over was accelerated in streptokinase therapy(260).

In another study, Tytgat et al.(261)found that patients with cirrhosis of the liver had a shortened fibrinogen half-life due to chronic disseminated intravascular coagulation. Fibrinogen half-lives were normalized with heparin therapy(261).

Zetterqvist(262) found the half-life of fibrinogen in patients with coagulation deficiency states to be within the normal range. In polycythemia vera, fibrinogen catabolism was abnormally rapid, as well as in some cases of liver cirrhosis and fatty liver. Intravascular coagulation was suspected as the cause. Patients with epidermolysis bullosa had normal fibrinogen half-lives(262).

Ansari and Silvis(263) applied the plasma concentration of fibrinogen to the differential diagnosis of an enlarged and irregular liver(263). Patients were classified by fibrinogen concentration into five groups: 1. normal, 2. miscellaneous diseases, 3. Laennecs cirrhosis, 4. malignant tumor, and 5. metastatic diseases(263).

3. Dosimetry:

Kakkar(278) reported that the injection of 100 μCi of I-125 fibrinogen will deliver a radiation dose of 200 mrem to the blood, 20 mrem to the tissues and 4 mrem to the kidneys. In addition, he reported that I-125 fibrinogen does not cross the placenta, but free I-125 was present in small quantities in fetal tissues. Radioactivity was also found in breast milk(278).

EXPERIMENTAL

I. MATERIALS

A. Animals:

Mongrel dogs of either sex weighing 10-20 kg were used in the experimental measurement of plasma clearance rates of I-125 labelled fibrinogen. They were individually housed in large cages with water ad libitum, and fed twice daily (Dr. Ballard's Champion Dog Food). All the urine was collected and stored until the radioactivity could be safely discarded.

The rabbits used in the human fibrinogen plasma clearance studies were mature New Zealand white rabbits, weighing 4.0-4.1 kg. They were individually housed with free access to food and water.

B. Chemicals:

All chemicals used in these investigations were of A.C.S., reagent, U.S.P. or B.P. specifications.

The following chemicals were purchased from Fisher Scientific Co. Ltd., Fair Lawn, New Jersey:

1. Trichloroacetic acid M. W. 163.39
2. Potassium oxalate M. W. 184.24
3. Iodine M. W. 126.90
4. Tham^{R} - tris (hydroxymethyl) aminomethane M. W. 121.14
5. Glycine M. W. 75.07
6. Cysteine hydrochloride monohydrate M. W. 140.19
7. Sodium Citrate M. W. 294.10
8. Potassium iodide (granular) M. W. 166.01

9. Aluminum hydroxide M. W. 155.99
10. Sodium phosphate dibasic heptahydrate M. W. 268.07
11. Sodium iodide M. W. 147.02
12. Sodium carbonate 105.99
13. Sodium hydroxide M. W. 40.01
14. Urea M. W. 50.06
15. Phenol Reagent

The following chemicals were obtained from other sources:

1.	Sodium chloride M. W. 58.45	MacArthur Chemical Co. Montreal, Quebec
2.	Nembutal Sodium M. W. 248.26	Abbott Laboratories Ltd. Montreal, Quebec
3.	Innovar-Vet	McNeil Laboratories (Canada) Don Mills, Ontario
4.	E-Toxa-Clean	Sigma Chemical Co. St. Louis, Mo.
5.	Anticoagulant Citrate Dextrose Solution U.S.P. Formula A (contained in plastic bags for the collection of 450 ml of blood)	Fenwal Laboratories Morton Grove, Ill.
6.	Monochloroacetate M. W. 94.5	B. D. H. Chemicals Ltd. Poole, England
7.	Sodium Dichromate M. W. 298	B. D. H. Chemicals Ltd. Poole, England
8.	Sulfuric Acid, Concentrated M. W. 98.08	MacArthur Chemical Co. Montreal, Quebec
9.	Methanol M. W. 32.04 99.5% CH_3OH	MacArthur Chemical Co. Montreal, Quebec
10.	Ethanol M. W. 46.07 95% C_2H_5OH	MacArthur Chemical Co, Montreal, Quebec

11.	Sodium Hydroxide M. W. 40.01 Standard Volumetric Solutions 1.0 N and 0.1 N	Anachemia Chemicals Ltd. Montreal, P. Q.
12.	Hydrochloric Acid M. W. 36.46 Standard Volumetric Solutions 1.0 N and 0.1 N	Anachemia Chemicals Ltd. Montreal, Quebec
13.	Sodium Thisosulfate M. W. 158.13 Standard Volumetric Solution 0.1N	Anachemia Chemicals Ltd. Montreal, Quebec
14.	ε- Aminocaproic acid M. W. 131.17	Fluka, A. G. Switzerland
15.	Potassium Phosphate Monobasic M. W. 136.09	New York, N. Y. Allied Chemical Co.
16.	β- Alanine M. W. 89.10	J. T. Baker Chemical Co. Phillipsburg, N. J.
17.	Sterile Water for Injection	Baxter Laboratories of Canada Ltd. Malton, Ontario
18.	Sodium Chloride Injection U. S. P.	Cutter Laboratories Ltd. Calgary, Alberta

C. Sodium Iodide-125:

Sodium iodide-125 was obtained from International Chemical and Nuclear Corporation of Montreal, Quebec in 10 mCi amounts, dissolved in 0.03 ml of 0.1 N NaOH pH 11.5. The radionuclidic purity was stated to be greater than 99%. Being protein reagent grade, the I-125 was carrier free and reductant free. This material was diluted to 0.2 ml with 0.1 N NaOH and refrigerated until used.

D. Chromatographic Material:

Sephadex^{R} is a gel filtration material supplied as dried beads by Pharmacia (Canada) Ltd. The product is prepared by cross-linking

dextran by means of epichlorohydrin to produce a three-dimensional matrix. Sephadex beads swell in the presence of water, due to the presence of hydrophilic hydroxyl groups.

Sephadex G-10 has a dry particle diameter of 40-120 μm, a water regain value of 1.0 ml of water per gram of dry beads, a bed volume of 2-3 ml per dry gram and a fractionation range of 0-700 daltons.

Sephadex G-25 medium has a particle diameter of 50-150 μ, a water regain value of 2.5, a bed volume of 4-6 ml/g, a fractionation range of 1000 to 5000 daltons.

Sephadex G-200 has a particle diameter of 40-120 μ, a water regain value of 20 ml/g, a bed volume of 30-40 ml/g and a fractionation range of 5000 to 800,000 daltons.

Sephadex G-10 and G-25 are converted to a gel by heating a weighed portion of dry beads in excess distilled water on a steam bath for at least 1 hour. Sephadex G-200 was prepared in a similar manner, except that it was heated for at least 6 hours.

Sepharose^{R} gel filtration media, supplied by Pharmacia(Canada) Ltd., is made from agarose, a linear polysaccharide of alternating residues of D-galactose and 3,6 anhydro-L-galactose units. The material is supplied in liter bottles of hydrated spherical agarose gel beads, and is ready for use without further preparation.

Sepharose 2B contains 2% agarose. The particle size is 60-250 μ, and the exclusion limit for proteins is 40×10^6 daltons.

Sepharose 6B is supplied as 40-210 μ beads with an agarose content of 6%. The exclusion limit is 4×10^6 daltons for proteins.

Disposable columns of Sephadex G-25 medium were obtained from Pharmacia (Canada) Ltd. These prepacked columns have a bed volume

of 9.1 ml and a void volume of approximately 2.5 ml for proteins. These columns were used to produce sterile, pyrogen-free fibrinogen in the following way: The columns were disassembled and autoclaved, followed by reassembly in a laminar flow hood. The columns were repacked with sterile, pyrogen free saline, followed by equilibration with sterile, pyrogen-free isotonic phosphate buffer, pH 7.4.

Dowex 1x-4 anion exchange resin was obtained from BioRad Laboratories, Richmond, California. This is a weak anion exchange resin, which consists of beads of polystyrene matrix with quaternary functional groups in the chloride form. It was prepared by equilibrating the beads in 0.1N HCl for a few minutes, then washing the beads to neutrality with distilled water. For sterile, pyrogen-free fibrinogen production, the beads were autoclaved in distilled water, and packed in a sterile disposable 5 ml syringe equippped with a Swinnex adapter containing a 0.8 μ membrane filter (Millipore Corp.). This was used in the removal of iodide and iodate from labelled fibrinogen reaction mixtures.

D. Chromatographic Material:

Electrophoresis and immunoelectrophoresis was carried out on an ACI Casette Electrophoresis Cell and Power Supply, using ACI agarose Universal Electrophoresis Film, supplied by Analytical Chemists, Inc., Palo Alto, California. Barbital buffer pH 8.6, 0.05 M with 0.035% EDTA was used in all analyses. One microliter samples were chromatographed for 30 minutes. Proteins were fixed and stained after electrophoresis or diffusion by immersing the film

in 0.2% Amido Black in 5% acetic acid for 15 minutes. The film was destained by agitating in 5% acetic acid for 30 seconds. It was then dried at 75°C for about 15 minutes, followed by immersion in 5% acetic acid for 1.0 minute, and repeated in fresh acetic acid for 1.0 minute. The film was dried at 75°C for 15 minutes.

Anti-human serum (Hyland Division of Travenol Laboratories, Inc., Costa Mesa, California) was allowed to diffuse for 48 hours before staining the film as indicated above.

E. Freeze-Dried Human Fibrinogen:

Freeze-Dried Human Fibrinogen was obtained in 1 gram bottles as a gift of the Canadian Red Cross Society. The protein was fractionated from plasma by Connaught Laboratories Ltd. (Toronto, Ontario) using the Cohn Cold Ethanol Fractionation Procedure and lyophilized. It was refrigerated until used.

Human Fibrinogen (U.S.P.) was also obtained from Cutter Laboratories, Berkley, California in a 1 g bottle. It also contained 0.92 g Na citrate, 2.5 g dextrose, 60 mg glycine.

F. Solutions:

All solutions were made with de-ionized and double distilled water. The solutions were filtered through 0.45 μ membrane filters (Millipore Corporation, Bedford, Mass.) and stored in closed containers at room temperature.

1. Isotonic Buffered Diluting Solution pH 7.4 was made according to the following formula:

KH_2PO_4	1.9 g
Na_2HPO_4	8.1 g
NaCl	4.11 g
H_2O q.s. ad	1.0 l

2. Tris Buffer pH 8.0

Tris	6.05 g
HCl 2N	13.4 ml
H_2O q.s. ad	1.0 l

3. A series of buffers were made according to the U.S.P. XVII, except that the buffers were made 0.2 N in NaCl. The pH was determined by the use of a Beckman Zeromatic pH meter, which was standardized with Beckman Standard Buffer pH 7.0 (Beckman Instruments, Inc., Fullerton, California). The following stock solutions were used:

(i)	KH_2PO_4	27.218 g
	NaCl	11.688 g
	H_2O q.s. ad	1.0 l
(ii)	NaOH	0.2 N
	NaCl	13.6 g
	H_2O	0.5 l

4. Potassium Iodide Carrier Solution:

A stock solution of 3×10^{-2} M KI was made by dissolving 498 mg of KI in sufficient water to make 100 ml. One ml of stock solution was then diluted with sufficient buffer of the desired pH to make 100 ml of 3×10^{-4} M KI.

5. Thrombin:

Topical Thrombin (Bovine Origin) was supplied by Parke, Davis and Co. in 10,000 unit vials. The enzyme was dissolved in 20 ml of normal saline and divided into 1.0 ml aliquots

and kept frozen until used.

6. Alkaline Urea Solution:

Alkaline Urea solution was prepared by dissolving 40 grams of urea in sufficient 0.2 N NaOH to make 100 ml.

G. Electrolytic Cells for Radioiodination of Fibrinogen:

Macro-Electrolyte Cell

For the initial studies of the parameters involved in electrolytic radioiodination of human fibrinogen, a cell similar in design to that reported by Khalkhali was used(63). The anode consisted of a 30 ml platinum crucible, which was the working electrode on which the oxidation of iodide to iodine occurred. The cathode (passive electrode) was a 12 cm long, 0.8 mm diameter platinum wire. This wire was contained in the cathode compartment, which consisted of a glass tube (10 cm x 1 cm) which was closed at the lower end by a dialysis membrane (Fisher Scientific Ltd., Fair Lawn, N. J.) with 48 Å pore size. A saturated calomel electrode was used as a reference electrode. The cell was supported by a Lucite case, which also supported the electrical connections. Figure 1 shows the details of the above cell, built by Mr. C. Ediss, Faculty of Pharmacy.

Micro-electrolytic cell

A smaller cell was designed for iodinations of small quantities of fibrinogen. This cell consisted of a block of polytetra-fluoro-ethylene (PTFE) into which was drilled a flat-bottomed hole, 1 cm deep and cm in diameter. The block was then divided in half in the plane of the axis of the cylindrical hole. A dialysis

Figure 1: Macro-Electrolytic Cell

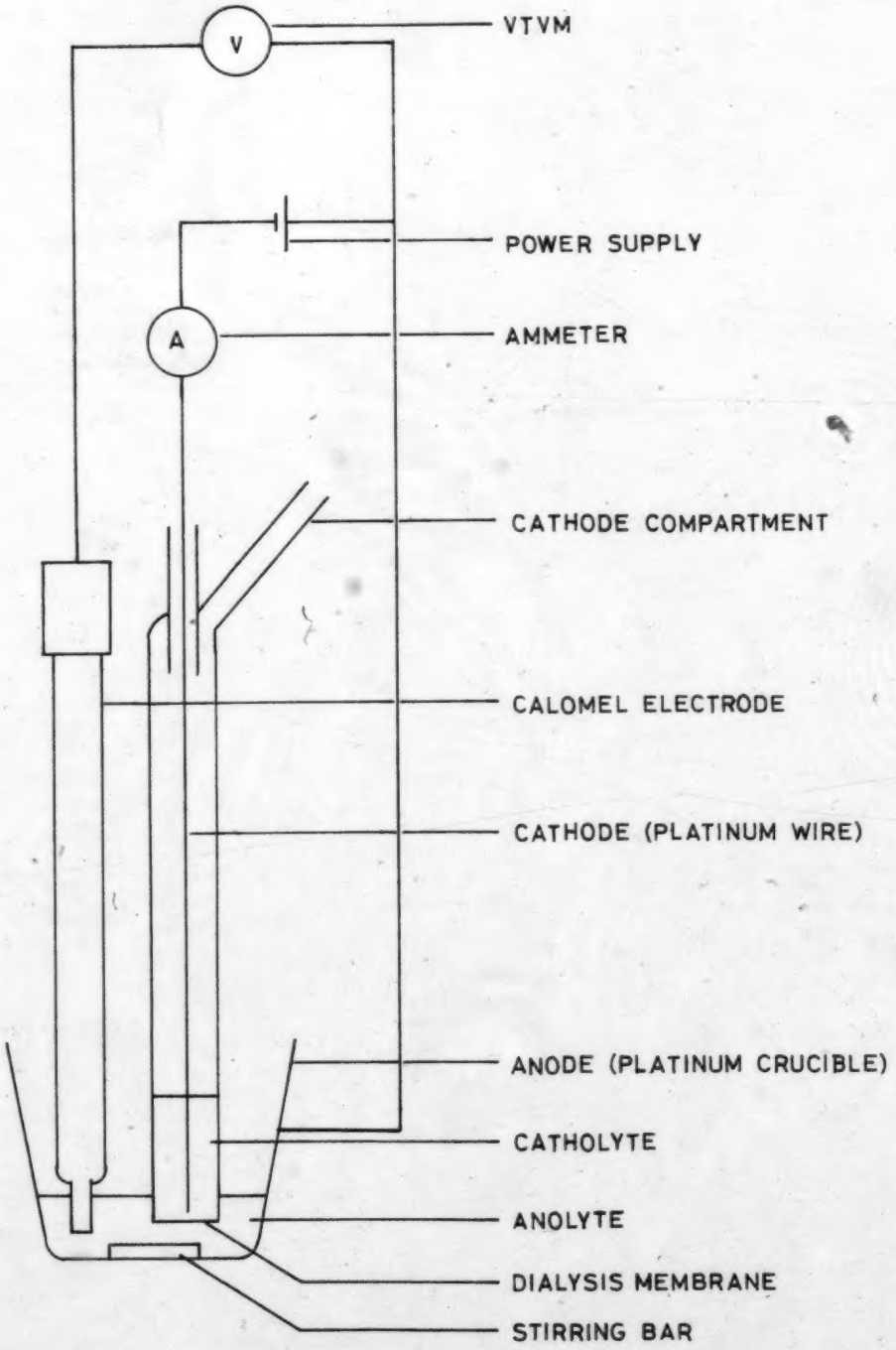
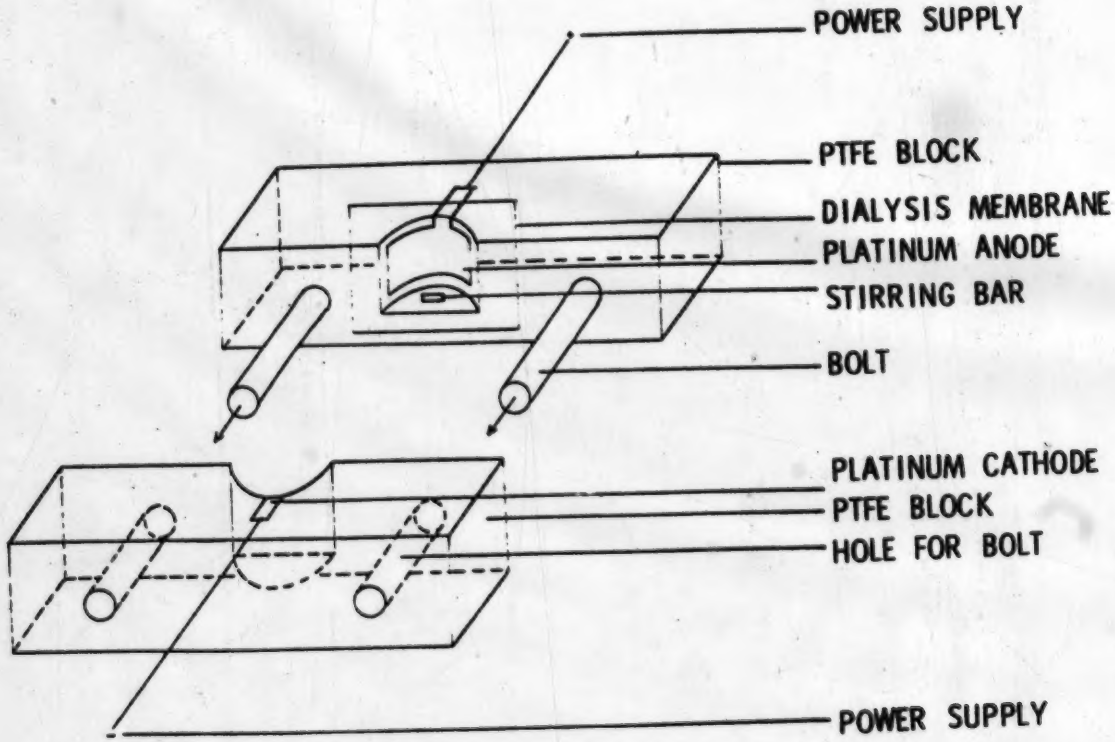


Figure 2: Micro-Electrolytic Cell



membrane could then be placed between the two pieces, which were then bolted together, forming two separate compartments. The wall of each compartment was lined with a piece of platinum foil (0.001" thick) to which electrical connections were made. One compartment was the anode and the other the cathode; each contained approximately 0.6 ml. Figure 2 shows the details of the above cell.

H. Electronics:

The power for the electrolytic cells was supplied by two 1.5 volt "D" dry cell batteries in series with a ten-turn potentiometer. For constant current iodinations, a similar power source was used, except that the current was maintained at a constant value by a transistorized circuit. A voltmeter (Armaco Multi Range D. C. voltmeter, Model SM 331, Armaco, Japan) was used to measure the applied voltage between the anode and cathode (Pt-vs Pt), and a vacuum tube voltmeter (Heathkit Model IM-18, Heath Company, Benton Harbor, Michigan) was used to measure voltage between the saturated calomel electrode and the anode. A microammeter (Armaco Multi-Range DC microammeter, Model SM 301, Armaco, Japan) was connected in series to measure the current flow through the cell as illustrated in Figure 1.

II. METHODS

A. Isolation of Fibrinogen from Plasma:

Several methods were used for the isolation of fibrinogen from plasma. These methods are described below.

Method 1

Blood was collected from the ante-cubital vein of healthy volunteers into six citrated (3.8%, 0.5 ml) Vacutainers (Becton-Dickinson & Co. Canada Ltd., Clarkson, Ontario) with 4.5 ml draw. The anticoagulated blood was centrifuged at 2000 rpm (840 g) for 15 minutes on an International Equipment Corporation - CM centrifuge and the plasma was then removed and subjected to chromatography on a Sephadex G-200 column (5 cm x 90 cm) each at 4°C. The column itself was a K 50/100 column obtained from Pharmacia (Canada) Ltd., and was equipped with two flow adapters, so that upward flow could be used to prevent excessive packing. The eluting buffer was Tris buffer pH 8.0 and the flow rate was 1.0 ml per minute. The eluate was monitored by means of a flow through ultra-violet photometer (UV Monitor, Pharmacia (Canada) Ltd., Montreal, Quebec). Based on the work of Lewis(274), the first of three peaks was collected and allowed to warm to room temperature. Powdered glycine (165.15 g/litre) was added to the eluate and dissolved with very slow stirring on a magnetic stirrer according to the method of Kazal(13). The solution was then centrifuged in polycarbonate centrifuge tubes (Nalge Company, Rochester, New York) for 25 minutes at 0°C and 31,000 g in a Servall Refrigerated Centrifuge equipped with an SS-1 head. The precipitated fibrinogen was collected and dissolved in the desired buffer. The solution was then clarified by passing it through a 0.45 micron membrane filter (Millipore Corp., Bedford, Mass.), and stored at 4°C until used. Figure 3 shows the elution profile obtained by the chromatographing of human plasma on Sephadex G-200.

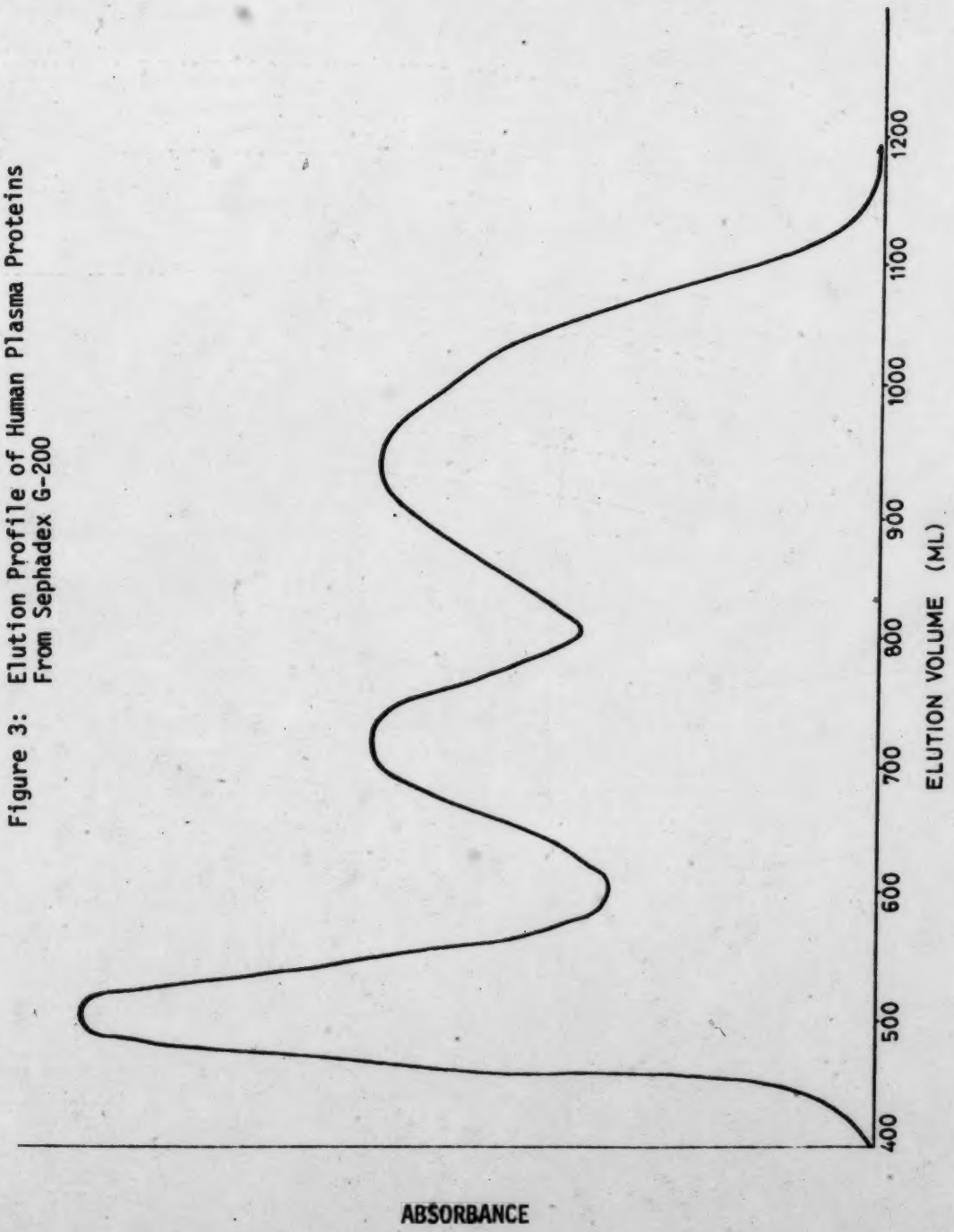


Figure 3: Elution Profile of Human Plasma Proteins From Sephadex G-200

Method 2

Venous blood (11ml) was collected into a 10 ml heparinized syringe and the plasma was separated by centrifugation at 10,000 rpm at 0°C for ten minutes. Five to six ml of plasma was separated and 165 mg of powdered glycine was added per ml of plasma and dissolved with slow stirring. The suspension was centrifuged at 16,000 rpm (31,000 g) at 0°C for twenty minutes. The precipitate was dissolved in 1.0 ml of tris buffer pH 8.0 and chromatographed on a Sephadex G-200 column (17 cm x 2.6 cm), using tris buffer for elution. The column a (K26/40 column obtained from Pharmacia Canada Ltd.) was equipped with two flow adapters, so that upward flow could be used. The flow rate was 0.3 ml/minute and the procedure was carried out at 4°C. The eluate was monitored spectrophotometrically by means of a UV monitor and the first peak was collected in a volume of 15-20 ml. One half the volume of the eluate of 3 M ammonium sulfate was added slowly to the eluate to precipitate the protein by the method of Hagen et al.(137). The precipitated fibrinogen was separated by centrifuging the suspension at 16,000 rpm at 0°C for twenty minutes. The protein was then dissolved in 1.0 ml of the desired buffer and clarified by filtration through a 0.45 micron membrane filter.

Method 3

Venous blood was collected into two citrated Vacutainers 0.5 ml 3.8% sodium citrate, 4.5 ml draw) and was spun at 2,000 rpm for 15 minutes at ambient temperature. Five-six ml of plasma

was separated and 0.3 ml of 25% ε-amino-caproic acid (EACA) was added. Aluminum hydroxide gel, 0.5 ml of 50% $Al(OH)_3$, was added, and the plasma was incubated at 37°C with agitation for five minutes. The plasma was filtered through a 0.45 micron membrane filter, and adsorbed once more with aluminum hydroxide as before.

The adsorbed plasma was then made 2.7 M in beta-alanine by slowly adding a solution of 6 M beta-ananine with stirring, according to the method of Jacobsen and Kierulf(165). Fibrinogen was separated by centrifuging the plasma at 30,000 g for 10 minutes at 15°C. The supernatant was discarded, and the precipitate was washed with 2.7 M beta-alanine .The protein was dissolved in 4.5 ml of pH 7.0 phosphate buffer and then 0.3 ml of EACA was added. Fibrinogen was reprecipitated with 6.0 M beta-alanine and the supernatant discarded. The precipitate was washed with 2.7 M beta-alanine and with pH 7.0 buffer, before dissolving the pellet in 0.7 ml of pH 7.0 phosphate buffer. The solution was clarified by passing it through a 0.45 micron membrane filter.

A total of thirteen samples of human fibrinogen isolated by Method III using sterile and pyrogen free equipment were radioiodinated by the electrolytic method, and the unbound iodide was removed by passing the labelled fibrinogen through a Dowex 1X-4 ion exchange column or through a Sephadex G-25 column. The eluting buffer was pH 7.4 isotonic phosphate buffer. The buffered fibrinogen solution was filtered through 0.22 micron membrane filters (Millipore Corporation) into sterile, pyrogen free multi-dose vials. The

samples were collected into 4 lots, which were forwarded independently for sterility and pyrogen testing. Aerobic and anaerobic cultures and cultures for fungi were held for two weeks; the procedures were carried out in the laboratories of Dr. F. L. Jackson, Department of Medical Bacteriology, University of Alberta. Aliquots of the 4 lots were also tested in rabbits for the presence of pyrogens; the testing was carried out under the direction of Dr. D. M. Lyster at the Vancouver General Hospital, Vancouver, B.C.

Method 4

Canine fibrinogen was isolated by the method given in Method 1 for human fibrinogen. Canine plasma was obtained from donor dogs at the Surgical Medical Research Institute, University of Alberta.

Method 5

Canine blood was obtained at the Surgical Medical Research Institute, from a dog exsanguinated after undergoing surgery. The blood was collected into one-tenth volume of potassium oxalate (0.1M) and was spun at 10,000 rpm for 10 minutes at 4°C. The plasma was separated and centrifuged again at 10,000 rpm for 10 minutes at 4°C. Epsilon amino-caprioic acid (EACA) was added to make the plasma 0.1 M in EACA. A solution of 6 M beta-alanine was added slowly to the plasma, until the final concentration was 2.7 M(165). The precipitated fibrinogen was removed by centrifuging

at 30,000 g for 10 minutes 15°C. The supernatant plasma was discarded and the fibrinogen was washed twice with pH 7.0 buffer, and redissolved in 150 ml of the same buffer. The solution was made 0.1 M in EACA and 6 M beta-alanine was added slowly to make the solution 2.7 M in the amino acid. The concentration of EACA was increased to 0.1 M, and the solution was stirred for 30 minutes. The fibrinogen was removed by centrifuging for 10 minutes at 30,000 g and 15°C. The precipitate was dissolved in 45 ml of pH 7.0 phosphate buffer (μ = 0.3) and filtered through a 0.8 micron membrane filter into plastic vials and stored at -20°C.

B. Quality Control of Sodium Radioiodide-125

Radionuclidic Purity:

The radionuclidic purity of the stock solution was confirmed by obtaining gamma energy spectrum by means of a NaI(Tl) crystal detector, storing in a multichannel analyzer (Northern Scientific, Middleton, Wisconsin) and recording the data on an x-y plotter. The two peaks observed were the result of the 27.4 KeV and 35.5 KeV single emissions and the 55 KeV coincidence emissions of I-125.

Radiochemical Purity:

The radiochemical purity of sodium iodide-125 was determined by chromatographing a diluted aliquot of the stock solution on a column of 75% G-25 Sephadex and 25% G-10 Sephadex, which was found to fractionate iodide from iodate. The eluate was pH 7.4 isotonic phosophate buffer. The eluate was collected in frations and

assayed for radioactivity.

C. Electrolytic Radioiodination of Fibrinogen

1. A General Procedure for Electrolytic Radioiodination of Fibrinogen

Fibrinogen was dissolved in 0.05 M phosphate buffer containing not less than 0.15 M NaCl and ranging in pH from 6.0 to 8.0. The desired volume of potassium iodide (3×10^{-4} M) in the same buffer was added, together with an appropriate quantity of sodium iodide - 125. Volumes of this solution, known as the anolyte, ranging from 0.3 to 3.0 ml were placed in the anode compartment of the electrolytic cell. The cathode compartment was filled with 0.3 to 4.0 ml of the supporting buffer.

The power supply was connected across the cell, and microammeter and voltmeter were connected by appropriate wiring. The saturated calomel electrode was used with the large cell by dipping the KCl bridge into the anolyte; the potential between it and the anode was measured by the use of the vaccum tube voltmeter (VTVM). Current was passed through the cell for a period of 15 or 30 minutes.

After each electrolytic radioiodination, the electrodes were washed in soapy water, and soaked in a chromic acid bath for a short period of time before rinsing thoroughly in distilled water. The dialysis membrane was discarded after each iodination.

2. Methods of Determining the Yield of the Radioiodination Procedure

a. Paper Chromatography:

Ten microliter aliquots were removed from the reaction mixture and spotted on Whatman No. 1 paper*and dried. The chromatograms were developed in 85% methanol and were allowed to run for approximately 20 centimeters. The strips were then cut into 1 cm segments and assayed for radioactivity on a Nuclear Chicago 1195 gamma spectrometer (W & R Balson Ltd., England).*

b. Trichloroacetic Acid Precipitation:

Ten microliter aliquots were removed from the reaction mixture and diluted to 1.0 ml with saline. A few drops of 25% albumin were added, and 1.0 ml of 20% trichloroacetic acid was added. The suspension of denatured protein was centrifuged for ten minutes at 2000 rpm. The total radioactivity was assayed, and the supernatant was then removed, and the activity of the precipitate and the supernatant were then determined.

c. Gel Filtration Fractionation:

A column of G-25 Sephadex was prepared by plugging the outlet of a 10 ml disposable syringe with a small amount of glass wool. Hydrated Sephadex G-25 was poured into the column to fill it to the 10 ml mark, followed by 30 ml of pH 7.4 isotonic phosphate buffer. Fifty microliter aliquots of the reaction mixture were applied to

the top of the column and eluted with the pH 7.4 buffer. The column fractionated the mixture into a I-125 labelled fibrinogen and an iodide-iodate fraction.

3. Gel Permeation Chromatography:

Fifty microliter samples of the reaction mixture were also chromatographed on a column of 75% Sephadex G-25 and 25% Sephadex G-10. The column was a K 16/20 column (Pharmacia Canada Ltd.) 1.6 cm x 13 cm; the eluting buffer was pH 7.4 isotonic phosphate buffer; the flow rate was 2.0 ml per minute and the void volume was 9.2 ml. This procedure separated the reaction mixture into three fractions: the first contained labelled fibrinogen, the second contained iodate-125, and the third contained iodide-125. The eluate from the column was collected in 6.0 ml aliquots and assayed for radioactivity on a Picker Autowell II gamma spectrometer.

The eluate was monitored as it passed through a thin polyethylene cannula placed in front of a shielded 3" NaI(Tl) crystal detector which was appropriately connected to a Canberra Model 1417 B Spectroscopy Amplifier, a Canberra Model 1481 L Lin/Log Ratemeter, A Canberra Model 456 High Voltage Supply and a Canberra Model 1437 Timer S. C. A. (Abtec Engineering, Ottawa). All results were recorded on a Beckman 10" Recorder (Beckman Instruments, Fulteron, California).

4. The Parameters of Electrolytic Radioiodination of Fibrinogen

The principal factors influencing the yield of I-125 fibrinogen by the electrolytic method were considered to be the following: 1. the amount of current flowing through the cell, 2. the voltage applied across the cell, 3. the volume of anolyte, 4. the volume of catholyte, 5. the concentration of carrier iodide in the anolyte, 6. the concentration of supporting electrolyte in the anolyte, 7. the pH of the anolyte, 8. the concentration of the protein in the anolyte, 9. the temperature of the reactants, and 10. the technique of electrolysis. The method by which these factors were studied is given below.

a. The Effect of Constant Current on Iodination Yield

In this study, the anolyte consisted of 3.0 ml of 2×10^{-5} M KI, 16.9 mg of protein (Cutter) in pH 8.0 buffer (0.05 M tris, 0.15 M NaCl). The cathode was filled with 2.0 ml of supporting buffer. The current was applied at a constant intensity for thirty minutes; the following amperages were examined: 35 uA, 75 uA, 150 uA, and 300 uA.

b. The Effect of Anolyte Volume on Iodination Yield

The yield of labelled fibrinogen was determined when the total anolyte volume was decreased from 3.0 ml to 2.0 ml and 1.0. This was achieved by decreasing the volume of supporting

electrolyte (pH 7.0, 0.05 M phosphate, 0.152 M NaCl, μ = 0.3), but maintaining the quantities of Connaught Fibrinogen (20.4 mg) and potassium iodide (0.2 ml of 3.0×10^{-4} M KI stock solution) constant. The current was maintained at 75 uA for 30 minutes.

c. The Effect of Concentration of Carrier Iodide

The yield of labelled fibrinogen was measured when the initial concentrations of KI in the anolyte were varied throughout the range of 1.2×10^{-4} M, 6×10^{-5} M, 3×10^{-5} M, 1.5×10^{-5} M, 7.5×10^{-6} M, to 3.75×10^{-6} M. The anolyte volume was 1.0 ml and contained 20.4 mg of protein in pH 7.0 phosphate buffer. A constant current of 50 uA and a voltage maximum of 0.8 vs the saturated calomel electrode (SCE) was passed for 15 minutes.

d. The Effect of Concentration of Protein in the Anolyte

The yield of labelled fibrinogen was measured when the protein concentration in the anolyte was varied from 6×10^{-5} M to 7.5×10^{-5}M. The iodide concentration was maintained at 1.5×10^{-5} M in pH 7.0 phosphate buffer and the anolyte volume was 1.0 ml. The current was 50 uA and the maximum voltage was 0.8 volt vs SCE; the reaction time was 15 minutes.

e. The Effect of the Volume of Catholyte on Reaction Yield

Iodination yield of labelled fibrinogen was measured when the volume of catholyte 9pH 7.0 phosphate buffer) was decreased

from 4.0 to 1.0 ml. The anolyte volume was kept constant at 1.0 ml, the protein concentration was 6×10^{-5} M, and the iodide concentration was 1.5×10^{-5} M. The current was 50 uA and the maximum voltage was 0.8 volt vs SCE; the reaction time was 15 minutes.

f. The Effect of pH on the Iodination Yield

The pH of the electrolyte was varied from 6.0 to 8.0 and consisted of 0.05 M phosphate buffer containing 0.15 M NaCl. The anolyte contained 20 mg of protein in a volume of 1.0 ml, and the iodide concentration was 3×10^{-5} M. The cathode was filled with 2.0 ml of the same buffer. The current was supplied at 1.0 volt for thirty minutes.

The effect of applied voltage maximum on the yield of iodinated fibrinogen was determined by passing a constant current of 50 uA through the cell until the desired applied voltage maximum was obtained. This ranged from 0.6 to 1.2 volts. The

anolyte contained 20 mg of protein, 3×10^{-6} M KI, in pH 7.0 phosphate buffer, while the cathode contained 4.0 ml of buffer.

h. The Effect of Supporting Electrolyte

The effect of removing sodium chloride from the electrolyte was tested by electrolytically radioiodinating Connaught fibrinogen in pH 7.0 phosphate buffer (μ = 0.148) and comparing the yield of labelled fibrinogen with that obtained when the above buffer contained 0.152 M NaCl and μ = 0.3. One ml of anolyte contained 6×10^{-5} M protein and 3×10^{-5} M potassium iodide. The catholyte consisted of 4.0 ml of the appropriate buffer, and the current was 50 uA and the maximum voltage was 0.8 volt vs SCE; the reaction time was 15 minutes.

i. The Effect of Temperature on Iodination Yield

The effect of lowering the reaction temperature to approximately zero degree Centrigrade was carried out by dissolving fibrinogen (20.4 mg) in pH 7.0 phosphate buffer (0.30), mixing in carrier iodide (1.5×10^{-5} M) and cooling the anolyte to 0°C. The catholyte (pH 7.0 phosophate buffer) was also cooled by placing in ice water. One ml of anolyte was placed in the anode compartment, and 4.0 ml of the catholyte in the cathode compartment. The anode was surrounded by ice water, while 50 uA of current and a maximum

of 0.8 volt vs SCE was passed for 15 minutes.

j. The Effect of Labelling by Constant Current, Constant Potential, and Constant Current-Constant Potential Methods

An anolyte consisting of 20 mg of protein and 1.5×10^{-5} M KI in pH 7.0 phosphate buffer and a total volume of 1.0 ml was electrolyzed for 30 minutes by three different methods: 1. constant current of 50 uA, 2. a constant cell voltage of 0.8 volt vs SCE, 3. 50 uA current until a voltage of 0.8 volt vs SCE is reached. The catholyte volume was 4.0 ml and the reaction was carried out at ambient temperature.

E. Methods for Analysis of the <u>In Vitro</u> Properties of Electrolytically Radioiodinated Fibrinogen

1. Quantitative Analysis:

A method of quantitative analysis of the fibrinogen content of the Commerical products was required since the one gram vials of freeze-dried human fibrinogen contained several adjuvants, including 5-10 g of glucose per vial. They were initially assayed by determination of nitrogen content, assuming a protein nitrogen content of 16%(105). Connaught fibrinogen was then used as a standard in a modification of a Folin-Phenol procedure(267) to produce a standard curve of absorbance versus fibrinogen concentration. The quantity of isolated fibrinogen could therefore be measured by reference to the standard curve.

However, it was found that this procedure was too time

consuming so the method of Blomback(12), which measures the absorbance of a solution of fibrinogen in 0.2 M NaOH - 40% urea was used. This procedure required the incubation of samples for at least 1 hour before determination of absorbance.

It was found that the method of Dellenback(268), which measures the optical density of fibrinogen solutions in 0.005 M Na Citrate was the most convenient due to its simplicity and absence of an incubation period. This procedure was found to be sufficiently accurate for the purpose required, and substitution of 0.9% saline for 0.005 M sodium citrate did not introduce a significant error. The assay of total protein in fibrinogen preparations supplied by Cutter and Connaught was made by determination of nitrogen in weighed portions of each product by means of a Coleman Nitrogen Analyzer (Coleman Instruments Corp., Maywood, Illinois).

Isolated fibrinogen was initially quantitated by the following Folin-Ciocalteau procedure(267): 10 mg of lyophilized Connaught fibrinogen was dissolved in 5.0 ml of saline and five aliquots (0.2 to 1.0 ml) of this solution were diluted to 1.0 ml with distilled water. One ml of saline was included as a blank. One ml of 10% NaOH was added to each solution, were then placed in a boiling water bath for ten minutes. Next, 7.5 ml of distilled water was added to each hydrolyzate and the blank followed by 0.5 ml of phenol reagent (Fisher Scientific Co., Fair Lawn, N. J.) and 3.0 ml of 20% (w/v) sodium carbonate. The solutions were then incubated for 30 minutes. The absorbance of the solutions were read at 650 nm against

the blank, and a standard curve of the absorbance versus weight of fibrinogen was prepared. Aliquots of the fibrinogen solution freshly isolated from plasma were treated in the same manner, and the absorbance measured against the same blank at 650 nm. The concentration of fibrinogen was then determined by reference to the standard curve.

Fibrinogen was also quantitated by measuring the absorbance of aliquots dissolved in sufficient 0.2 M NaOH and 40% urea to make 4.0 ml against a blank of the same solution, and comparing the absorbance with the extinction coefficient for fibrinogen $E^{1\%}_{1cm}$ = 16.17(12).

Fibrinogen was also assayed by diluting aliquots to 4.0 ml with saline, and measuring the absorbance of the solution against a saline blank in a Beckman DU spectrophotometer. The concentration of the fibrinogen solution was calculated by means of the absorbance coefficient for fibrinogen $E^{0.1\%}_{1\ cm}$ = 1.55(268).

2. Assay of Factor XIII

The presence of factor XIII in samples of fibrinogen was tested by noting the solubility of clotted fibrinogen in 1% monochloroacetic acid for 24 hours.

3. Assay of Plasminogen

Fibrinogen isolated by Method I was tested for the presence of plasminogen by the method of Bishop(267), at the University of Alberta Hospital, under the direction of Dr. J. R. Hill.

4. Sedimentation Velocity Analysis

Sedimentation velocity analysis of a 0.3% solution of human fibrinogen prepared by method I dissolved in 0.15 M NaCl was carried out on a Beckman Model E Analytical Ultracentrifuge with Schilieren optical system (single sector cell with 4° - 12 mm Kel-F centerpiece, and quartz windows) and was recorded on a Kodak metallographic plate. The analysis was carried out by Mr. M. Aarbo in the laboratory of Dr. C. Kay in the Department of Biochemistry, University of Alberta.

5. Disc Electrophoresis

Human fibrinogen was isolated by Method I and labelled electrolytically with I-125 and subjected to disc electrophoresis. A solution of 36 mg% fibrinogen in pH 7.0 buffer was diluted with 80% glycerol and chromatographed on large pore gels (5% acrylamide) using a Canalco Model 1200 Analytical Disc Electrophoresis Bath, by the method Grabar(275). The upper and lower buffer was pH 8.5 tris-glycine - EDTA (0.001 M). A current of 3.5 mA per tube was applied for a two hour period. The gels were stained with Coonassie Brilliant Blue R stain and destained with 7% acetic acid. The gels were then cut into 1.5 mm sections and analyzed for radioactivity. The disc electrophoresis was performed by Mr. E. Browne in the laboratory of Dr. R. Bridger in the Department of Biochemistry, the University of Alberta.

6. Sepharose Chromatography

Native and electrolytically radioiodinated fibrinogen were chromatographed on a Sepharose 2B column (20 cm x 2.5 cm) and a Sepharose 6B column (34 cm x 2.5 cm). The eluting buffer was pH 7.4-0.2 M phosphate - 0.07 M NaCl buffer and the elution rate was 1.0 ml per minute. The eluate was monitored continuously at 280 nm by a UV Monitor (Pharmacia Canada Ltd.) and the results were recorded graphically on a 10" chart recorder (Pharmacia Canada Ltd.) The area under the elution profile curve was determined by cutting the figure out and weighing it, and comparing the weight with that of a rectangle of similar known area made from the same paper.

If labelled fibrinogen was chomatographed, the entire anolyte mixture was applied to the columns without further processing. The eluted fractions were collected and 6 ml aliquots were assayed for radioactivity on a Picker Nuclear Autowell II gamma spectrometer. The columns were back washed with the same buffer, and the back-wash was collected and counted in the same manner.

7. Clottability

The clottability of native and electrolytically radioiodinated fibrinogen was determined spectrophotometrically by the following method: The absorbance (A_1) of fibrinogen solutions containing 0.5 to 2.0 mg/ml in pH 6.3 phosphate buffer (μ = 0.15) was determined by diluting aliquots to 4.0 ml with buffer, and measuring the absorbance against a blank of the same buffer at 280 nm. Twenty five N.I.H. units of bovine thrombin (Parke-Davis, Montreal) were

added to the original fibrinogen solution, and two hours later the clot was removed by winding on an applicator stick or by expressing the clot liquor from the clot by passing it through a 0.45 micron membrane filter. The absorbance (A_2) of an aliquot of the clot liquor was measured after dilution to 4.0 ml with pH 6.3 buffer against a blank of the same buffer. Clottability was calculated as $(A_1 - A_2)/A_1 \times 100)$.

8. Thrombin Time

The thrombin time of native and radioiodinated preparations of Connaught Fibrinogen were determined by adding 0.1 ml of the fibrinogen solution of 15 mg/ml in pH 7.0 phosphate buffer ($\mu = 0.3$) to 0.1 ml of 0.05 M $CaCl_2$ and warming for at least one minute at 37°C, after which 0.05 ml of thrombin (500 NIH units /ml) was added. The time to visible gelation was recorded for three different preparations of the labelled protein, which had an average protein to iodine ratio of 3.7:1.0.

Thrombin times were also determined in three samples of labelled and unlabelled fibrinogen isolated by method III. Aliquots (0.2 ml) of the preparations (6.1 mg/ml in pH 7.0 phosphate buffer, $\mu = 0.3$) were warmed to 37°C for at least 1 minute, after which 0.1 ml of thrombin (15 NIH units per ml) were added and the time to visible gelation was determined. The protein-to-iodine ratio of the material was 1:1.6.

9. Stability of the Label

The rate of hydrolysis of I-125 from electrolytically labelled samples of fibrinogen was measured by incubating labelled Connaught fibrinogen and fibrinogen isolated by method II at 37°C and measuring the percent of radioactivity bound to the protein at 24-hour intervals by chromatographing 50 µl samples on a Sephadex G-10+G-25 column. The freshly isolated fibrinogen was incubated in pH 7.0 buffer, while the labelled Connaught fibrinogen was incubated in pH 7.0 buffer, 1.0×10^{-3} M cysteine, 6% albumin, and in human serum.

F. Methods for Measurement of the <u>In Vivo</u> Properties of Electrolytically Radioiodinated Fibrinogen

1. Clearance of Labelled Canine Fibrinogen in Dogs

Six dogs of either sex weighing 10-20 Kg were injected with 300 µCi of canine fibrinogen labelled with I-125 by the electrolytic method. Two animals received fibrinogen isolated by Method 4, which was injected immediately after isolation and labelling. Four dogs received fibrinogen isolated by Method 5 which was frozen and stored at -20°C before use.

Five minutes after the injection of the preparation, the first blood samples were collected into 2.5 ml heparinized syringes, using 26 x 1/2" needles. Samples were then taken 10, 15 and 30 minutes, then half-hourly for the first three hours, then hourly for the next six hours and then twice daily for 13 days. This procedure

allowed collection of an adequate volume of blood without alteration of the animal's circulatory physiology; the small needle allowed repeated punctures of fore-limb veins without causing hematomas or phlebitis.

Blood samples were centrifuged and 0.5 ml plasma samples were assayed for radioactivity on a Searle 1195 gamma spectrometer. These results (the $\log_{10}$ of counts per minute versus time in hours) were analyzed by a stripping method on a Digital PDP-11 computer.

Aliquots of plasma samples were diluted with 1 ml of saline and mixed with 1.0 ml of 20% trichloroacetic acid and were centrifuged at 2,000 rpm for 10 minutes. The samples were assayed for radioactivity in the precipitate and the supernatant, and the TCA insoluble fraction was expressed as a percent of the total radioactivity in the sample.

2. Rabbit Clearance Rates of Labelled Human Fibrinogen

The following study of the clearance rate of electrolytically labelled human fibrinogen was performed under the direction of Mr. J. R. McLean at the Radiation Protection Bureau, Division of Radiation Medicine in Ottawa, Ontario.

Human Fibrinogen was isolated by Method 3 and radioiodinated by the electrolytic method. The labelled protein was chromatographed on a column of G-25 to remove free iodide. The solution was frozen and packed in dry ice and shipped by air to Ottawa, Ontario, where the studies described below were carried out.

The marginal ear vein of three rabbits weighing 4-4.1 kg were cannulated and 30 μCi of I-125 human fibrinogen were injected. Blood

s were collected at 15 minutes, 4 hours, 24 hours, 48 hours
hours. Two 25 microliter samples were counted to at least
counts. A composite curve of results was plotted by $y = \log_{10}$
tial activity remaining) vs the time in hours. The curve was
red manually.

Tenth ml aliquots of the 15 minute and 4 hour samples were
itated with 1.0 ml of 10% TCA and centrifuged at 4°C for 20
s at 2000 rpm. Plasma samples of the same time periods were
lectrophoresed on polyacetate strips (Gelman) at 25-30 mA/
and at 250-300 volts for 60-90 minutes.

3. Imaging of Surgically Induced Thrombi in Leg Veins of Dogs

Venous thrombi were induced in the veins of the hind legs
grel dogs weighing 10-20 Kg. Thrombi were induced by two
: 1. suturing a cotton thread soaked in bovine thrombin
nits/ml) into the long saphenous veins at the level of the
on, or by cauterizing the intima of the vein by passing a
a venocath through the saphenous vein for a distance of 11-13
the deep veins in the thigh and passing a cauterizing current
Cutting Coagulator) to the exposed segment (1.5 cm) of the

roximately 500 μCi of labelled fibrinogen I-125 were
ntravenously immediately after thrombus induction.
r scans, venograms, and multi-wire proportional chamber
4" field of view) were performed at selected intervals.
rform a rectilinear scan, the dog was lightly anaesthe-
tra-muscular injections of Nembutal Sodium and Innovar-

Vet, the dose being adjusted for the weight of the dog. The animal was placed on a table of the appropriate height and the leg was arranged to display a level surface to the scanning probe. The rectilinear scanner (Picker Magna-Scanner 500/D, Picker Corporation Cleveland,Ohio) was adjusted to detect the low energy emissions of iodine-125 and set at a low scanning speed. The detector head was set at a distance of 3 inches above the site of the induced thrombus. A large area of the leg containing the thrombus was scanned, the results being recorded on teledeltos paper and on x-ray film.

The multi-wire proportional chamber images of the thrombi were obtained by Dr. R. Snyder, Department of Bio-Medical Engineering, University of Alberta. This imaging device was designed and built by Dr. Snyder. Under light anesthesia the dog was placed on a table, and the leg containing the thrombus was placed directly on the detecting surface of the chamber, or in some cases, the chamber was placed on top of the leg. Fifty-thousand counts were accumulated by the equipment and the results were displayed on a cathode ray tube. A permanent record was made by photographing the CRT display.

Venograms were made under the direction of Dr. L. A. Davis, Department of Orthopedic Surgery, Faculty of Medicine, University of Alberta. They were obtained by the standard technique of intravenous injection of a radioopaque dye distal to the site of the induced thrombus and securing an x-ray when the dye had filled the venous system in the leg. The results were interpreted by Dr. Davis.

RESULTS AND DISCUSSION

I FIBRINOGEN

A. Measurement of the Yield of Radioiodinated Fibrinogen

1. Paper Chromatography:

Initially, ascending paper chromatography was used to separate radio-iodide and radio-iodate from labelled fibrinogen as previously described. It was found that when a shipment of NaI-125 was used for radioiodination shortly after it was received, the amount of radio-activity associated with fibrinogen on the chromatogram before beginning the electrolytic reaction was always less than 5%. However as the NaI-125 aged, the amount of activity associated with fibrinogen at the origin of the chromatogram increased drastically, thus increasing the uncertainty about the reliability of this separation method.

2. Trichloroacetic Acid Precipitation:

Although the TCA method of determining the yield of labelled fibrinogen is rapid, the resulting values were not as reliable as those obtained by chromatographic methods. Duplicate determinations tended to vary from each other and were generally higher than the values obtained by Sephadex chromatography. For example, the average difference between eleven duplicate determinations made by TCA precipitation was 5.7%. The mean percent of I-125 bound to fibrinogen before initiating electrolytic iodination was 8.8% in ten determinations. This was 5.6% higher than that obtained by Sephadex chromatography which were determined for different trials.

3. Gel Filtration Fractionation:

Fractionation of reaction mixtures through Sephadex-G-25 columns or a mixture of Sephadex G-25+G-10 columns produced the most accurate and reliable results, and were used where ever possible in this work. For example, the average difference between six duplicate determinations made by Sephadex gel filtration was 1.1% and the amount of I-125 associated with the fibrinogen fraction before beginning the iodination procedure was 3.2% in three trials.

B. Quality Control of Sodium Radioiodide-125

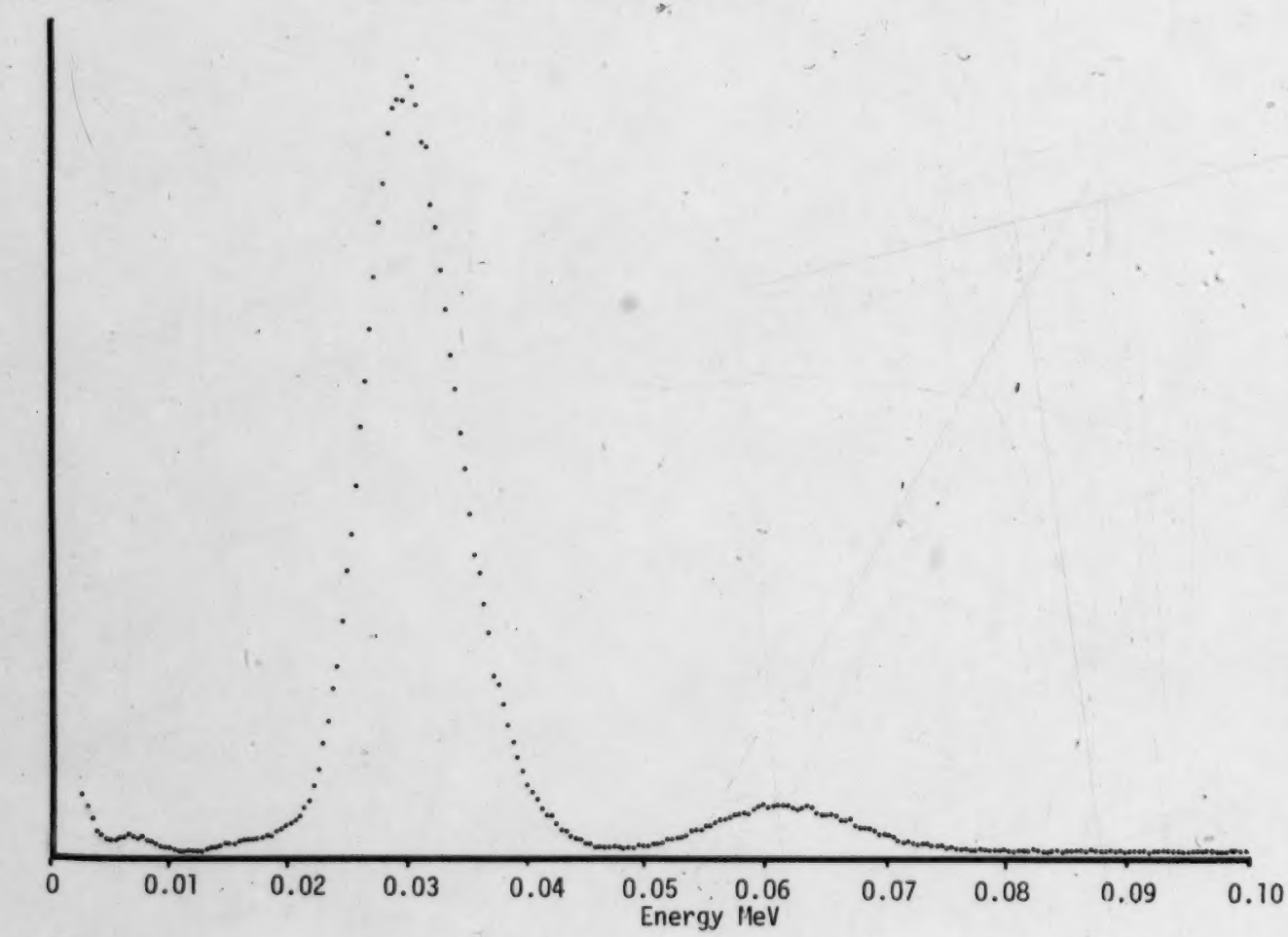
1. Radionuclidic Purity:

The energy spectrum of NaI-125 obtained by means of a NaI(Tl) crystal detector and multi-channel analyzer showed two peaks. The lower energy peak was centered at about 27 KeV and was the result of the 27.4 KeV and 35.5 KeV single emissions. The higher energy peak was centered about 55 KeV and was the result of the 55 KeV and 63 KeV coincidence emissions of I-125. No peaks of higher energy were observed. A spectrum of I-125 is shown in Figure 4.

2. Radiochemical Purity:

When a diluted aliquot of NaI-125 was chromatographed on a Sephadex G-10 +G-25 column to separate iodide and iodate, not less than 95% of the radioactivity was associated with the iodide peak.

Figure 4: ^{125}I Energy Spectrum Obtained from Sodium Iodide Scintillation Detector



C. Fibrinogen

The quality of the fibrinogen preparations (commercial and freshly isolated) used in the development of the radiopharmaceutical was tested by a variety of in vitro methods. The procedures carried out include quantitative analysis, clottability, disc electrophoresis, immunoelectrophoresis, agarose gel electrophoresis, gel chromatography sedimentation velocity analysis, and tests for the presence of Factor XIII and plasmin. Not all of these tests were performed on each of the preparations. A summary of the results of the quantitative analysis and of the clottability of the preparations is given in Table 6 .

1. Commercial Fibrinogen:

Difficulty was encountered in finding a commerically available fibrinogen which was sufficiently similar to freshly isolated fibrinogen to permit comparison of experimental values. Human Fibrinogen U.S.P. was initially obtained from Cutter Laboratories and was used to define electrolytic reaction parameters, but it did not compare well with isolated fibrinogen. It contained 28.2% by weight of fibrinogen, and was 77.5% clottable.

Human Fibrinogen was then obtained from the Connaught Laboratories, and was used throughout this study. It contained an average of 35.6% by weight of protein with a clottability of 88.7%.

TABLE 6

Clottability and Quantitative Analysis of Fibrinogen Preparations

Fibrinogen Preparation	Quantitative Analysis	S.D.	Plasma Volume (ml)[c]	S.D.	number of trials
Cutter	28.2% w/w[a]	-			1
Connaught	35.6% w/w	4.77			5
Method 1	31.9 mg[b]	10.3	18.3	0.64	6
	32.0 mg	10.8	18.3	0.64	5
	40.0 mg	8.8	30.0	3.6	8
Method 2	7.6 mg	1.25	5.5	0.53	8
Method 3	5.5 mg	1.76	5.2	0.68	15
Method 4	32.8 mg	16.9	32.7	10.2	5
Method 5	13.9 mg/ml		450		

(a) Percent by weight of protein content in preparation

(b) Weight of fibrinogen isolated from plasma

(c) Plasma volume from which fibrinogen was isolated

Fibrinogen Preparation	Clottability[d] %	Standard Deviation	Number of Trials
Cutter	77.5	2.8	3
Connaught	88.7	1.18	6
Method 1	95.1	2.48	18
Method 2	93.6	2.1	11
Method 3	95.8	1.5	12
Method 4	92.8	1.8	4
Method 5	96.3	0.72	3

(d) Percent of clottability protein as measured spectrophotometrically

2. Isolated Fibrinogen

a. Human

Method 1:

Fibrinogen isolated by Method 1 was subjected to quantitative analysis by several methods. The first method used was a colorimetric assay, modified from a method given by Langdell(266). This procedure gave an average value of 31.9 mg of fibrinogen from an average volume of citrated plasma of 18.3. The second method used the extinction coefficient of $E^{1\%}_{1cm}$ = 16.17, as determined by Blomback(12); this method yielded an average of 32.10 mg of fibrinogen for the same volume of plasma. The isolation procedure of Method 1 produced an average of 1.77 mg of fibrinogen per ml of citrated plasma.

It was found that these methods of quantitating fibrinogen were too time consuming, and therefore the method of Dellenback(268) was subsequently used. Fibrinogen isolated by Method 1 from 30.0 ml of citrated plasma yielded 40.0 mg as measured by this method. This was an average of 1.35 mg of fibrinogen per ml of citrated plasma, and is 0.42 mg/ml less than assayed by the previous methods.

The clottability of fibrinogen isolated by this method was 95.1%.Fibrinogen which was isolated by Method 1 was subjected to immunoelectrophoresis and run against anti-fibrinogen; the results indicated the presence of native fibrinogen. Samples were also run against anti-plasma and the results showed only one precipitin arc, indicating that the samples contained only fibrinogen.

When fibrinogen was subjected to disc electrophoresis, the

stained gels showed only a single band. Sedimentation velocity analysis of a 0.3% solution of the fibrinogen solution in normal saline demonstrated a symmetrical peak with a sedimentation coefficient of $S_{(obs)}$ = 6.75. There was also another peak of approximately 30 $S_{(obs)}$ and was estimated to be 3-4% of the total.

The preparation was found also to be free of active or available plasmin as determined by the method of Bishop(267). However, it does contain factor XIII, since clots were insoluble in 1% monochloroacetic acid after 24 hours. An overall assessment of this method indicated it would not be practical for use in the clinical application of labelled fibrinogen, due to length of the procedure, and inability to guarantee sterility against the hepatitis virus.

Method 2

Fibrinogen isolated by Method 2 yielded an average quantity of fibrinogen equal to 7.56 mg from a volume of 5.5 ml of plasma. This was a yield of 1.4 mg of fibrinogen per ml of plasma. The protein was found to be 93.6% clottable.

Because of the length of time required to sterilize and pack the chromatographic column, this method was also judged to be impractical from the stand point of routine preparation of autologous fibrinogen.

Method 3

Fibrinogen isolated by Method 3 yielded an average quantity of 5.5 mg of fibrinogen from a volume of 5.2 $\pm$ 0.16 ml. This

represents a yield of 1.05 mg of fibrinogen per ml of citrated plasma. The protein was dissolved in a volume of 0.83 ml of pH 7.0 buffer at an concentration of 5.24 mg/ml. The average clottability was 95.8%.

This material was chromatographed on Sephadex G-200 (21 x 2.6 cm). The eluting buffer was pH 7.4 isotonic phosphate buffer and the flow rate was 0.3 ml per minute. The eluate was monitored on a UV Monitor, and in the resulting electron profile the protein appeared in a single symmetrical peak at the void volume (33 ml). No lower molecular weight proteins were observed.

Method 3 fibrinogen was also chromatographed on Sepharose 6B column (35 x 2.6 cm). The eluting buffer was pH 8.0 tris buffer and the flow rate was 1.0 ml per minute. Native fibrinogen was eluted at 90 ml whereas a small peak of aggregates was eluted at 45 ml; this peak contained 5.5% of the total protein eluted.

This preparation was also subjected to electrophoresis and immunoelectrophoresis. It was electrophoresed with Connaught Fibrinogen and with human plasma for comparison. The Method 3 fibrinogen showed only a single stained band located at the same position of the major band found in the Connaught Fibrinogen. When run against anti-human serum, no precipitin arcs were visible.

In this procedure, all equipment used in the preparation of fibrinogen could be autoclaved and the procedure could be completed within two hours.

In summary, human fibrinogen prepared by Method 3 was free of contaminating proteins and was highly clottable. It could be isolated in two hours and the final solution was sterile and pyrogen-free. The aseptic proceudre did not require any special equipment that is not

readily found in hospital laboratories. Methods 1 and 2 cannot be applied clinically; however, Method 1 produced a high quality fibrinogen preparation in large quantity, as did the same method using canine plasma (Method 4). Beta-alanine cannot be used to isolate canine fibrinogen (Method 5).

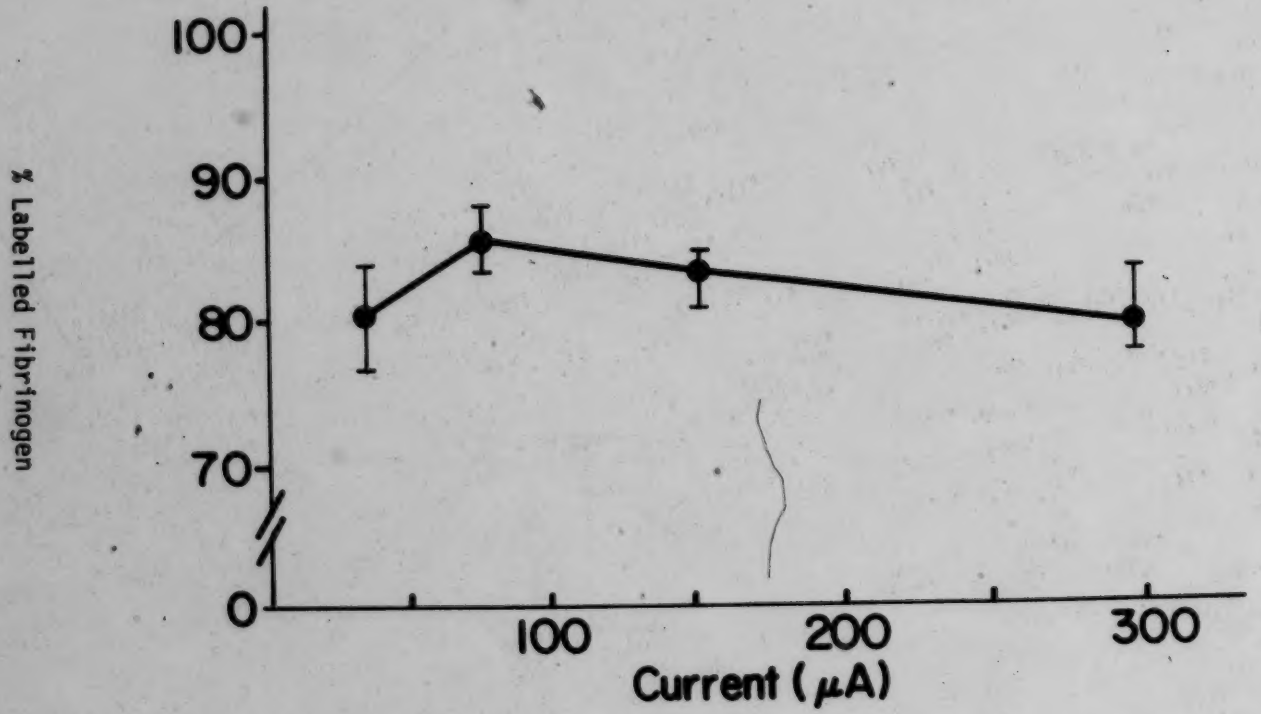
II. THE PARAMETERS OF ELECTROLYTIC RADIOIODINATION OF FIBRINOGEN

Each value obtained for a given set of conditions is the average of three trials, unless stated otherwise. In the graphical presentation of results, the mean of the values is given together with the range. In the tables of results, the average is given with the standard deviation and the standard error of the mean(SEM).

A. The Effect of Constant Current on Iodination Yield

For an anolyte volume of 3 ml, containing 2×10^{-5} M KI, 16.9 mg of protein in pH 8.0 buffer, the optimum current for maximum yield of labelled fibrinogen for the 30 minute time period was 75 μA. The optimum value was found to change directly with the volume of anolyte, ranging from 10 μA, for 0.3 ml, 50 μA for 1.0 ml, to 75 μA for 3.0 ml. The results are presented graphically in Figure 5 , and the average values are given in Table 7.

Figure 5: The Effect of Constant Current on Labelled Fibrinogen Yield



Each point represents the average of three trials. The bar indicates the range of results.

TABLE 7

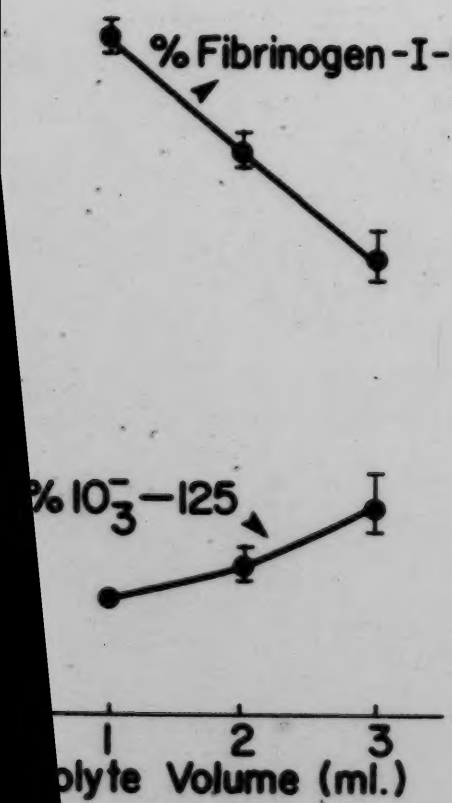
The Effect of Constant Current on Iodination Yield

Current μA	% Labelled Fibrinogen	S.D.	S.E.M.
35	80.2	3.42	1.71
75	85.6	2.37	1.37
150	83.2	2.42	1.40
300	78.7	1.40	1.84

B. Effect of Volume of Anolyte

The yield of labelled fibrinogen was measured when the volume of anolyte was decreased from 3 to 1 ml, but maintaining the quantities of protein (20.4 mg) and KI (0.2 ml of stock solution) constant. This has the effect of increasing the concentration of protein and carrier iodide by a factor of three, while maintaining the ratio of protein to iodide at 1:1. It was found advantageous to decrease the volume of anolyte to the minimum possible for a particular cell design to achieve the highest yield of labelled fibrinogen, and the lowest yield of labelled iodate. Iodate is undesirable for three reasons: 1. it cannot react with tyrosine to form a labelled product, and therefore, 2. a high yield of iodate is associated with a low yield of labelled fibrinogen, and 3. iodate forms a radiochemical impurity which must be removed before injection of the radiopharmaceutical. The results are presented graphically in Figure 6, and the average values are given in Table 8.

ure 6: The Effect of Anolyte Volume
on the Yield of Labelled Fibrinogen



ts the average of three trials.
he range of results.

TABLE 8

The Effect of Anolyte Volume on Iodination Yield

Anolyte Volume (ml)	% Labelled Fibrinogen	S.D.	S.E.M.	%Iodate	S.D.	S.E.M.
1	81.3	1.14	0.66	9.4	0.38	0.22
2	72.8	0.95	0.55	11.8	1.37	0.79
3	64.4	1.95	1.13	16.0	2.23	1.28

C. The Effect of Concentration of Carrier Iodide

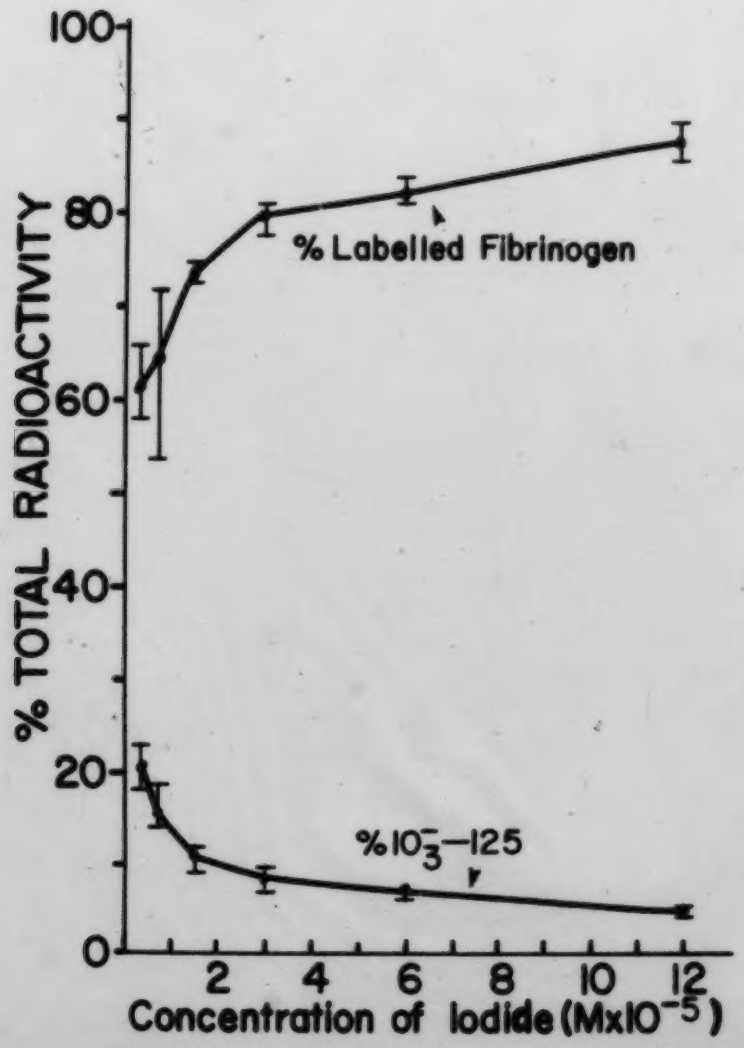
The effect of concentration of carrier iodide on iodination yield was measured by varying the iodide concentration over the range 3.7×10^{-6} M to 1.2×10^{-4} in 1.0 ml of anolyte containing 20.4 mg of protein at pH 7.4. The results in Figure 7 and Table 9 demonstrate that as the concentration of carrier iodide is decreased below 3×10^{-5} M, the yield of labelled fibrinogen decreases and the iodate formation increased.

TABLE 9

The Effect of Concentration of Carrier Iodide on Yield

Molarity of Iodide	% Labelled Fibrinogen	S.D.	S.E.M.	%Iodate	S.D.	S.E.M.
1.2×10^{-4}	86.77	2.1	1.21	3.9	0.4	0.25
6×10^{-5}	81.3	1.6	0.92	6.5	0.4	0.25
3×10^{-5}	79.6	2.2	1.09	8.3	1.2	0.58
1.5×10^{-5}	73.1	1.0	0.59	10.7	1.5	0.88
7.5×10^{-6}	64.1	9.4	5.4	15.8	2.5	1.46
3.75×10^{-6}	61.8	4.1	2.35	20.5	2.5	1.43

Figure 7: The Effect of Concentration of Iodide on the Yield of Labelled Fibrinogen



Each point represents the average of three trials.
The bar indicates the range of results.

D. The Effect of Concentration of Protein

The concentration of protein in the anolyte was varied over the range 6×10^{-5} M to 7.5×10^{-5} M, while maintaining the iodide concentration at 1.5×10^{-5} M in pH 7.0 phosphate buffer and a total volume of 1.0 ml. Figure 8 and Table 10 show that the yield of labelled fibrinogen tends to decrease below a concentration of 3×10^{-5} M protein, and iodate formation tends to increase as the concentration of protein is decreased.

TABLE 10

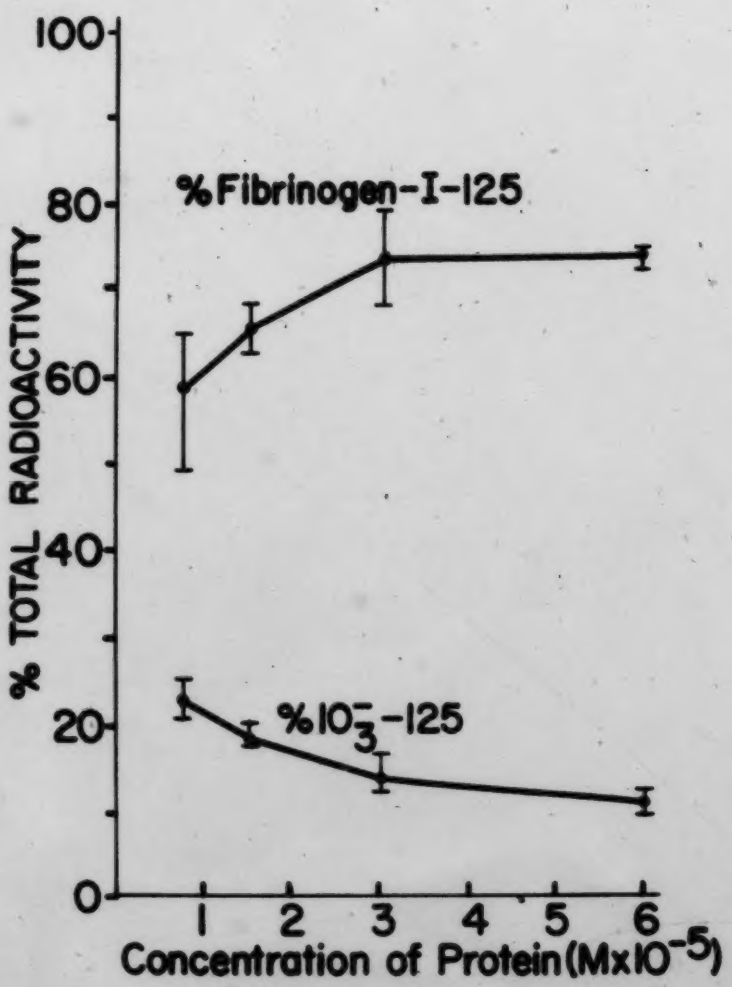
The Effect of Protein Concentration on Iodination Yield

Molarity of Protein	% Labelled Fibrinogen	S.D.	S.E.M.	% Iodate	S.D.	S.E.M.
6×10^{-5}	73.1	1.0	0.59	10.7	1.5	0.88
3×10^{-5}	73.5	5.5	3.19	14.2	1.9	1.10
1.5×10^{-5}	65.5	2.8	1.61	18.9	0.95	0.55
7.5×10^{-6}	59.2	6.9	3.09	21.4	3.1	1.37

E. The Effect of Volume of Catholyte

There was no appreciable difference in radioiodination yield when the volume of catholyte (pH 7.0 phosphate buffer) was decreased from 4.0 to 1.0 ml, while maintaining the anolyte volume constant at 1.0 ml, and the remaining parameters constant. The results are given in Table 11.

Figure 8: The Effect of Concentration of Protein on the Yield of Labelled Fibrinogen



Each point represents the average of three trials
The bar indicates the range of results.

TABLE 11

The Effect of Catholyte Volume on Iodination Yield

Catholyte Volume	% Labelled Fibrinogen	S.D.	S.E.M.	% Iodate	S.D.	S.E.M.
1	70.2	3.9	2.25	11.29	2.1	1.23
4	73.1	1.0	0.59	10.7	1.5	0.88

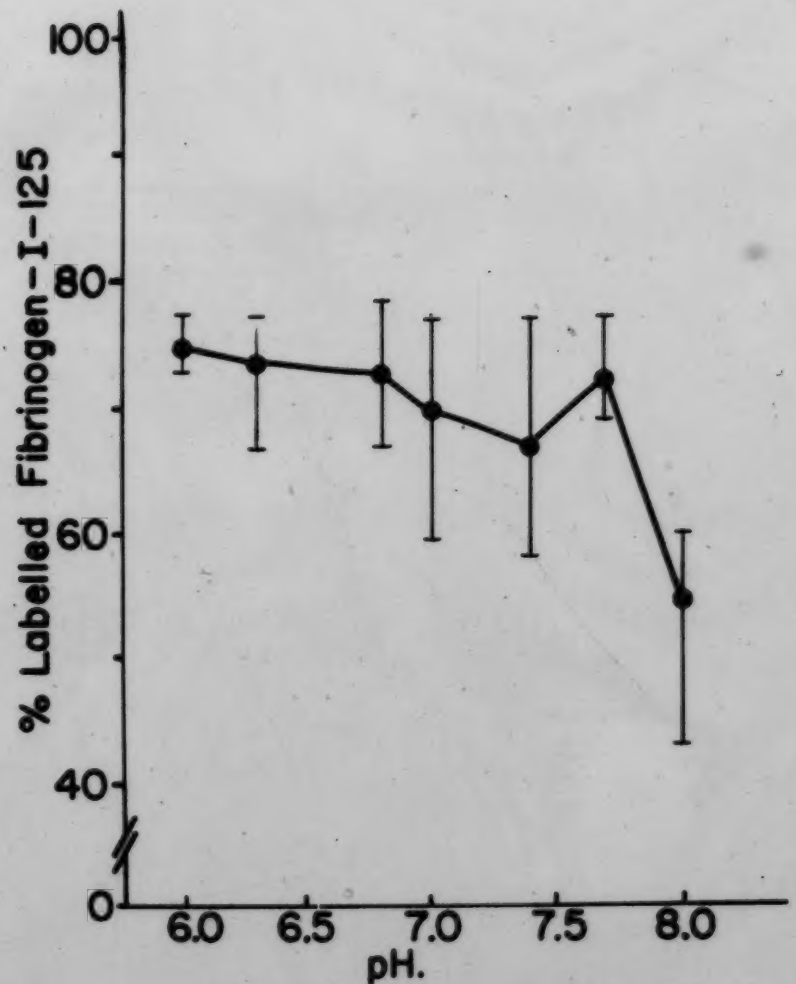
A catholyte volume of 1.0 ml requires a higher maximum applied voltage (1.64 v) than at 4.0 ml (1.03 v).

F. The Effect of pH on the Iodination Yield

The effect of pH on the yield of labelled fibrinogen was measured over the range of pH 6.0 to 8.0. The results shown in Figure 9 and Table 12 indicate a generally decreasing yield of labelled fibrinogen with increasing pH, and that the highest yield occurs at pH 6.0.

The studies of Hung(81) and Alexander(61,62) suggest that pH 7.0 is the most favorable pH for selective iodination of tyrosine and histidine, and for the least degradation of trytophane and cysteine. Scully(276) has found that the most even distribution of radioiodine among the alpha, beta, and gamma chains of fibrinogen is achieved at pH 7.0. In view of these studies, and since the yield of labelled fibrinogen at pH 6.0 (74.8%) is not significantly higher than that obtained at pH 7.0 (69.9%), the latter pH appears to be the most favourable for electrolytic iodination of fibrinogen.

Figure 9: The Effect of pH on the Yield of Labelled Fibrinogen



Each point represents the average of at least six trials. The bar indicates the range of results.

TABLE 12

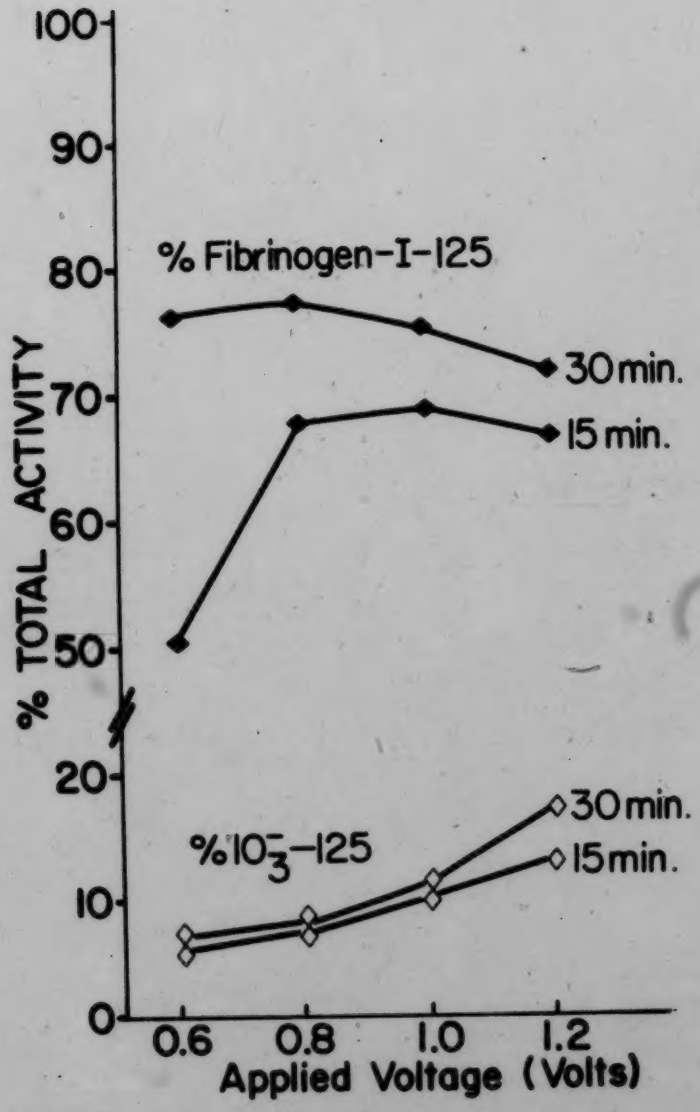
The Effect of pH on Iodination Yield

pH	% Labelled Fibrinogen	No. of Trials	S.D.	S.E.M.
6.0	74.8	6	1.8	0.73
6.3	73.8	7	3.7	1.41
6.8	72.7	9	3.8	1.28
7.0	69.9	7	5.9	2.21
7.4	66.7	6	6.2	2.53
7.7	72.0	7	3.2	1.19
8.0	54.0	7	5.9	2.24

G. The Effect of Applied Voltage Maximum on Iodination Yield

The effect of applied voltage maximum was determined by passing a constant current of 50 μA through the electrolytic cell, while allowing the voltage across the cell to rise to a desired maximum value. At that time, the current was allowed to decrease, while the voltage was maintained at the maximum value. This maximum voltage (Pt vs Pt) was varied over the range 0.6 volt to 1.2 volts. Figure 10 suggests that the optimum voltage for electrolytic iodination is between 0.8 volt and 1.0 volt. The percent of labelled fibrinogen and iodate obtained at 15 minutes and 30 minutes at various voltages is given in Table 13. One volt was chosen as the maximum applied (Pt vs Pt). This value is equal to 0.80 volts vs the

Figure 10: The Effect of Applied Voltage Maximum on the Yield of Labelled Fibrinogen



Each point represents the average of at least three trials. The Bar indicates the range of results.

saturated calomel electrode. Thus, when it was possible to use the saturated calomel electrode, a reference value of 0.8 volt vs SCE was used.

TABLE 13

The Effect of Applied Voltage Maximum on Iodination Yield

15 Minutes

Voltage Pt vs Pt	n	% Labelled Fibrinogen	S.D.	S.E.M.	% Iodate	S.D.	S.E.M.
0.6	6	50.1	7.3	2.99	5.8	1.29	0.74
0.8	11	67.1	9.9	2.99	7.3	2.03	0.91
1.0	6	68.7	2.1	0.86	10.0	1.63	0.94
1.2	4	66.4	3.5	1.72	2.6	1.46	0.73

30 Minutes

0.6	3	76.0	5.2	3.00	7.1	1.5	0.86
0.8	4	77.1	2.6	1.31	6.7	1.3	0.64
1.0	4	75.0	0.5	0.26	11.2	0.4	0.23
1.2	3	71.5	1.5	0.89	17.1	2.6	1.52

TABLE 14

Standard Reduction Potentials for Iodine Reactions(273)

		I_2	+ $2e^-$	⟶	$2I^-$			
		I_3^-	+ $2e^-$	⟶	$3I^-$			
IO^-	+	H_2O	+ $2e^-$	⟶	I^-	+ $2OH^-$		0.49
IO_3^-		$6H^+$	+ $5e^-$	⟶	$\frac{1}{2}I_2$	+ $3H_2O$		1.195
IO_3^-	+	$6H^+$	+ $6e^-$	⟶	I^-	+ $3H_2O$		1.085
$2IO_3$	+	$12H^+$	+ $10e^-$	⟶	I_2	+ $6H_2O$		1.19
IO_3	+	$3H_2O$	+ $6e^-$	⟶	I^-	+ $6OH^-$		0.56
IO_3	+	$3H_2O$	+ $6e^-$	⟶	I^-	+ $6OH^-$		0.26

By means of the Nernst Equation, a reversible decomposition potential can be calculated for the cell I^-/I_2 ($a = 3 \times 10^{-5}$)‖$Hg/HgCl_2$ (s) KCl (sat'd) assuming the cell resistance is negligible and that the anode and cathode over potentials are zero. The standard reduction potential for iodine reactions is given in Table 14. The decomposition potential for this cell is initially -0.5608 volt. For a cell to produce electrical work, there must be a decrease in free energy occurring in the cell, i.e., the change in free energy must be negative. Since $\underline{F} = nFE$, where n is the number of equivalents reacting, F is the Faraday and E is cell potential, and $\underline{F}$ is the free energy, E must be positive for a spontaneous reaction. Since the potential of the cell above is negative, the reaction is not spontaneous and at least 0.5608 volt must be applied to produce the

reaction. As the iodide is oxidized to iodine, the cell potential increases exponentially to -0.661 volt when the iodide concentration is 1% of the initial concentration.

H. The Effect of Supporting Electrolyte

The effect of sodium chloride as a supporting electrolyte was measured by determining the yield of labelled fibrinogen when the salt was absent from the anolyte, which then consisted of pH 7.0 phosphate buffer containing 6×10^{-5} M protein and 3×10^{-5} M KI. The yield was 77.2% labelled fibrinogen and 9.73% labelled iodate. This was not significantly different from the yield of labelled fibrinogen when sodium chloride was present: 79.6% labelled fibrinogen and 12.2% labelled iodate. Thus, there is no advantage conferred on the yield of labelled fibrinogen by increasing the concentration of sodium chloride. The results are given in Table 15.

I. The Effect of Temperature on Iodination Yield

The effect of lowering the temperature to approximately zero degrees centrigrade on the yield of labelled fibrinogen was carried out as previously described. The yield of labelled fibrinogen was 55.2% and the yield of labelled iodate was 13.6%. At ambient room temperature (approximately 21°C) and under the same reaction conditions, the yield of labelled fibrinogen was 73.1% and the yield of labelled fibrinogen was 10.7%. The results are shown in Table 16. This may be due to an increase in electrolyte resistance and an

increase in the transport number of iodide ions with decreasing temperature(277). While proteins are more stable at lower temperatures, the yield of labelled fibrinogen is significantly less than at ambient room temperature, and the protein will not be adversely affected at this temperature for short periods of time.

TABLE 15

The Effect of Supporting Electrolyte on Iodination Yield

NaCl Concentration	% Labelled Fibrinogen	S.D.	S.E.M.	% Labelled Iodate	S. D.	S. E. M.
0.152 M	79.6	2.2	1.09	12.2	1.17	0.61
0.0	77.2	4.76	2.75	9.7	2.2	1.26

TABLE 16

The Effect of Temperature on Iodination Yield

Temperature °C	% Labelled Fibinrogen	S.D.	S.E.M.	% Labelled Iodate	S.D.	S.E.M.
0	55.2	4.4	2.56	13.6	0.96	0.56
21	73.1	1.03	0.59	10.7	1.53	0.88

J. The Effect of Electrolytic Technique on Iodination Yield

In order to evaluate the effect of electrolytic technique on the production of labelled fibrinogen, the following experiments were performed. An anolyte consisting of 20 mg of protein and 1.5×10^{-5}

M KI in pH 7.0 phosphate buffer and a total volume of 1 ml was electrolyzed by three different methods:

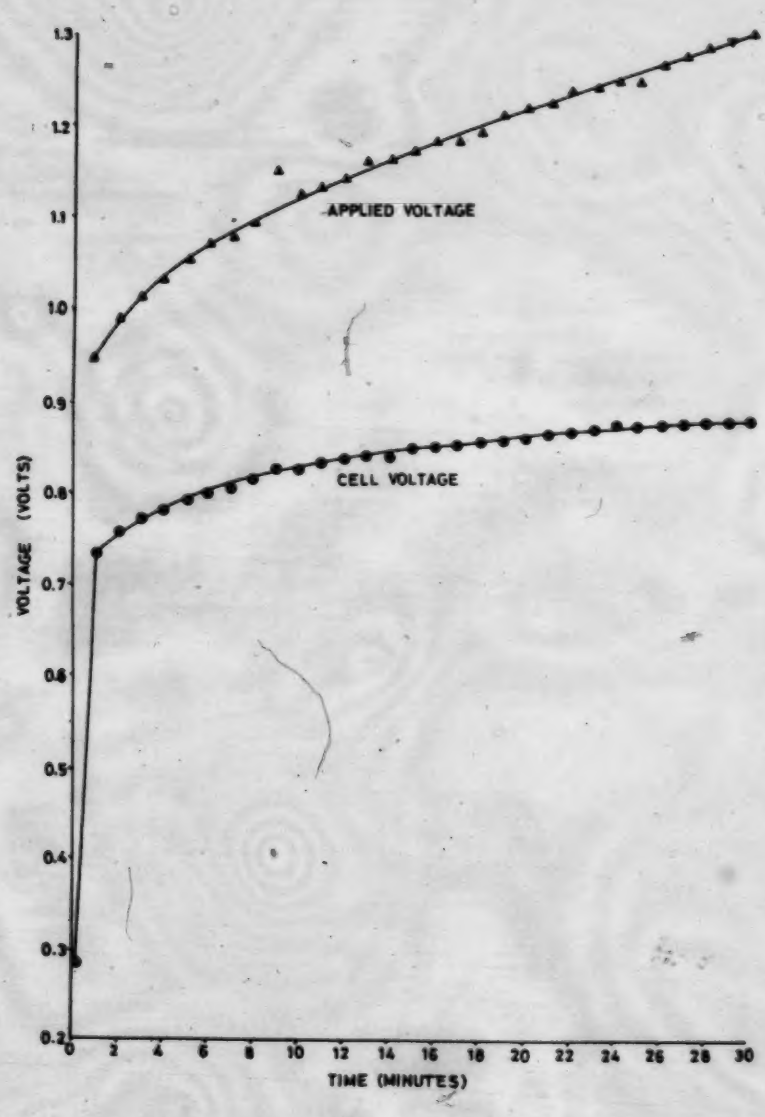
1. A constant current of 50 μA was passed through the cell while the cell potential (I^-/I_2 (a = 3 x 10^{-6})//Hg/Hg_2Cl_2(s), KCl sat'd) was allowed to rise freely. Figure 11 shows the increase in applied (Pt vs Pt) and cell potential with time at a constant current of 50 μA.

2. A constant, controlled voltage of 0.8 volt versus a saturated calomel electrode (SCE) was applied to the cell and the amount of current was uncontrolled. Figure 12 shows the decrease in applied potential and current with time at a constant potential of 0.8 volt versus the SCE.

3. A current of 50 μA was passed through the cell until the increasing cell potential reached 0.8 volt versus SCE. This potential was then maintained while the current decreased exponentially. Figure 13 shows the change in applied potential with constant cell potential, as well as the changes in current.
14 shows that there is a similar increase in cell potential and decrease in current with a maximum current of 50 μA followed by a maximum applied potential (Pt vs Pt) of 1.0 volt.

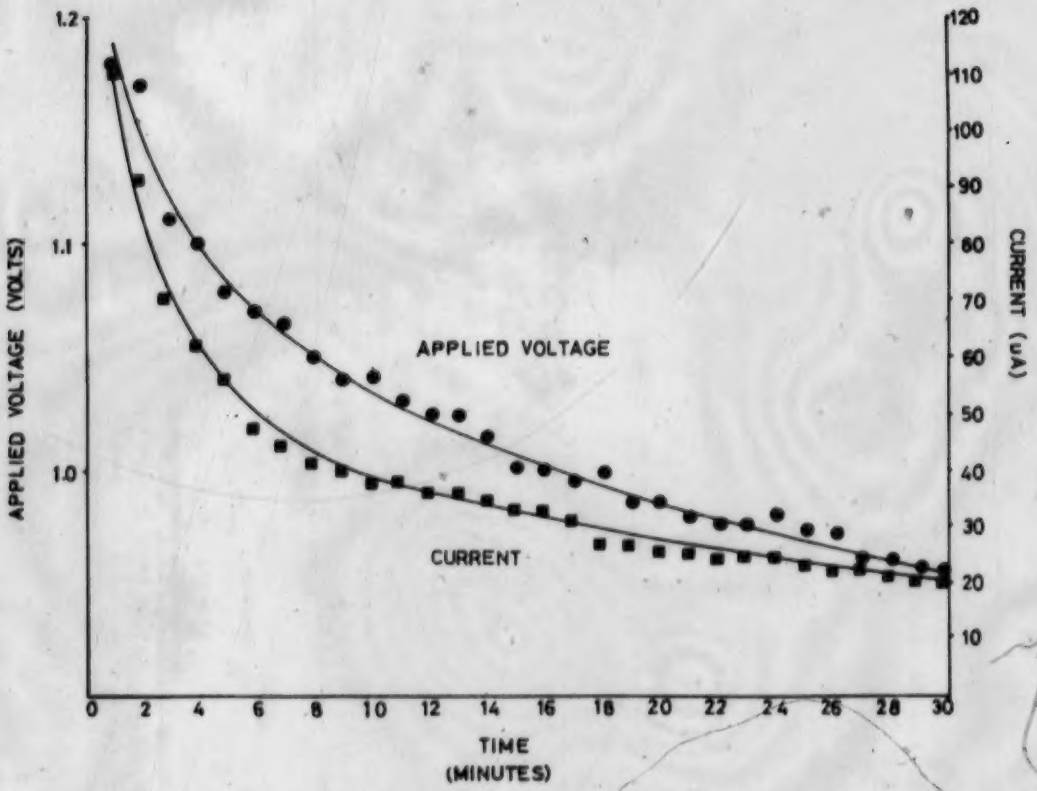
The average yield of labelled fibrinogen and iodate obtained in three trials of each of these techniques is given in Table 17 .

Figure 11: The Change in Cell Voltage and Applied Voltage of an Electrolytic Cell at Constant Current of 50 μA



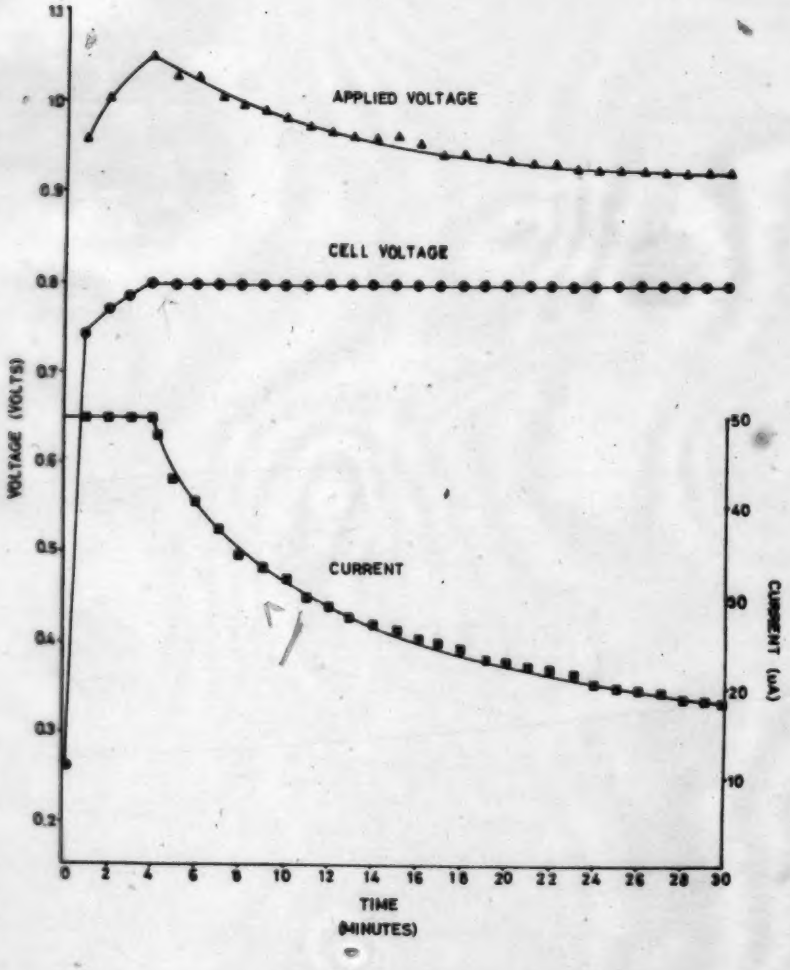
Each point represents the average of three trials.

Figure 12: The Change in Current and Applied Voltage of an Electrolytic Cell at a Constant Cell Voltage of 0.8 volt vs a Saturated Calomel Electrode



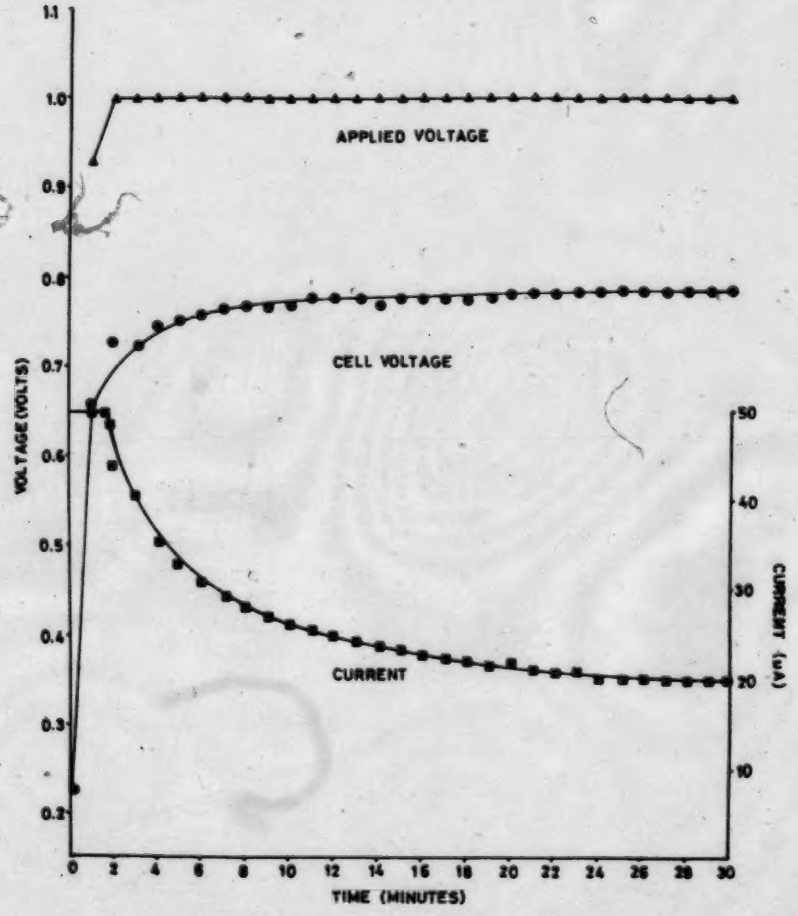
Each point represents the average of three trials.

Figure 13: The Charge in Current, Cell Voltage, and Applied Voltage when a Maximum of 50 μA and 0.8 volt vs SCE is Applied to the Electrolytic Cell



Each point represents the average of three trials

Figure 14: The Change in Current, Cell Voltage, and Applied Voltage When a Maximum of 50 μA and 1.0 volt (Pt vs Pt) is Applied to the Electrolytic Cell



Each point represents the average of three trials.

TO WHOM IT MAY CONCERN:

Page 179 is not missing but has been misnumbered.

TABLE 17

The Effect of Electrolytic Technique on Iodination Yield

Method	%Labelled Fibrinogen	S.D.	S.E.M.	% Labelled Iodate	S.D.	S.E.M.
50 μA	68.69	1.1	0.63	24.6	1.8	1.02
0.8 volt	62.4	5.3	3.07	30.1	2.7	1.54
50 μA + 0.8 volt	70.0	1.8	1.05	22.6	2.0	1.16

Table 17 shows that controlled current-controlled potential method produces a higher yield of labelled fibrinogen than does the constant voltage technique, but not the constant current. This is because the cell voltage (Pt vs SCE) did not exceed 0.80 volt. If a non-optimum current had been used, the yield would have been lower.

In the third technique, a current of 50 μA is applied until a potential of 0.8 volt vs SCE is attained, at which time the current falls exponentially to a residual current (Figure 12). If the value of the residual current (18.6 μA) is subtracted from each of the values of current when it is decreasing exponentially, a set of values is obtained which will yield a straight line when their logarithms are plotted linearly against time. This straight line fits the equation $\log_{10} y = ax + b$, where the slope a = 0.050377 and the ordinate intercept b = 1.4683, which is the log of 29.4. This line was fitted by the least squares method.

From the above equation, the time required for the current to decrease to one-half of a given value, the half time, is 5.975

minutes. This shows that the oxidation of iodide is a first-order process and the current at any instant is proportional to the concentration of iodide and to its rate of depletion. Thus, the time required to reach a particular degree of oxidation is independent of the initial concentration. The time required for the iodide to reach one-half of its original concentration is therefore 6 minutes. If electrolysis was begun at an initial current flow of 29.4 + 18.6 = 48 μA, it would require ten half-times or 60 minutes to decrease the concentration of iodide by 99.9%, and 30 minutes to oxidize 96.9% of the iodide.

Two types of electrolytic technique have been reported in the literature for electrolytic radioiodination: constant current electrolysis and constant potential electrolysis. The disadvantage of the constant current electrolysis is that the voltage required to supply a constant current increases beyond that required to oxidize iodine to that which produces large amounts of iodate, and may reach the potential required to oxidize the phenolic ring of tyrosine (slightly over 1.0 volt)(269). Other functional groups of proteins, such as imidazole, thioether, disulfide, and amine are also subject to electrolytic oxidation at this potential(269).

The disadvantage of controlled potential electrolysis for oxidation of iodide is that a high current is initially produced, which decreases exponentially with time according to the following equation(270):

$$i_t = \frac{nFACD}{\delta} e^{\frac{-DAt}{V\delta}} = i_o e^{-kt}$$

i_t - current at time t

n - number of equivalents

F - Faraday

A - electrode area

V - Volume of solution of concentration C

δ - diffusion layer thickness

D - diffiusion coefficient

i_o - initial current = $nFACD/\delta$

K = $DA/V\delta$

The reaction of iodine with tyrosine appears to be the rate limiting step in the electrolytic method of radioiodination of protein. Malan(128) has found that the time for iodination of thyrotrophin was about twenty times greater than the theoretical time for electrolysis of iodide to provide the same level of iodine.

The preceeding experiments indicate that there is a set of parameters for electrolytic radioiodination which must be optimized for each electrolytic cell in order to obtain the highest yield of labelled protein. For comparison, the parameters of electrolytic radioiodination procedures found in the literature are given in Table 18. It can be seen that the anolyte volume varied between a low of 10 µl,(Rollag-127) to a large volume of 15 ml (Teulings-121). A volume of 1.0 ml was found satisfactory in the experimental portion of this thesis. In Table 18, the current used ranged from 6-7 µA(122) to 1-2 mA(123). Sammon et al.(124) used a controlled

TABLE 18

Parameters of Electrolytic Radioiodination of Proteins and Hormones

Author	Current	Voltage	Electrolyte	Anolyte Volume	Iodide Conc. (M)	Protein Conc. (M)	Time	Yield %
Stevens (280)	50 μA	0.8 v (SCE)	PO_4-saline pH 7.4 21°C	1.0 ml	3×10^{-5}	3×10^{-5} fibrinogen	30 m	70-80
Caro (125)	20 μA		Ringer's PO_4 pH 7.4	5 ml		growth hormone 30-50 moles	2 h	
Donabedian (126)	5 μA		saline	60 l	C.F.	1-5 μg hormones	45	30-50
Harwig (132)	10-30 μA	0.4-0.5 (SCE)	barbital pH 7.4	8 ml	1.5×10^{-4}	fibrinogen 1.1×10^{-6}	1.5-4.5 h 4.5 h	
Javonovic (130)	1 mA		water pH 10	22 ml	1.3×10^{-3}	albumin 12%	4 h	90
Katz (123)	1-2 mA		PO_4-saline pH 7.5	10-15 ml		albumin 5.8×10^{-5} 6.8×10^{-5}	30-90 m	80-90
Khalkhali (63)	72-179 μA	0.4-0.7 v	saline pH 7.4	10 ml	3×10^{-5}	Fibrinogen 5×10^{-5}	30 m	85
Krohn (16)	70-300 μA	0.6 - 2.6 v	saline-PO_4 pH 7.8 4°C	7 ml	C.F.	Fibrinogen 4.2×10^{-7} 2.1×10^{-6}	70 150 m	30-80

TABLE 18(2)

Parameters of Electrolytic Radioiodination of Proteins and Hormones

Author	Current	Voltage	Electrolyte	Anolyte Volume	Iodide Conc. (M)	Protein Conc. (M)	Time	Yield %
Malan (128)	10 μA	PO_4 pH 7.4		30 μl	5×10^{-3} 1×10^{-2}	hormones 2-10 μg		
Pennisi (122)	30 μA		saline	3 ml	2.8×10^{-5}	insulin 1.2×10^{-4}	20 m	85-90
Pennisi	6-7 μA		saline 5°C		1.2×10^{-5}	insulin 8.6×10^{-6}	35-40 m	80-90
Rollag (127)		0.53 v (SCE)	PO_4 pH 7.1	10 μl	C.F. 6×10^{-10} moles	peptides $1\text{-}5 \times 10^{-10}$ moles	10 m	
Rosa (23)	100-500 μA		saline	10 ml	2.5×10^{-4}	fibrinogen 2.9×10^{-5}	30 m	73-77
Rosa (12)	300 μA	0.49 v	saline pH 7.5 8-10°C	8 ml		albumin 3.6×10^{-4}	12 h	
Sammon (124)		0.8 v (SCE)	PO_4-saline pH 7.5 4°C	1 ml	1.2×10^{-9} 2.4×10^{-9}	PTH 200-400 μg	20-30 m	86.5%
Teulings (121)	300 μA		saline pH 8.0	15 ml	2×10^{-5}	fibrinogen 1.2 x 10-5	9m	54%

potential of 0.8 volt vs the SCE and did not control the current. Harwig et al.(132), Khalkhali et al.(63), Rollag et al.(127), and Rosa et al.(120) used a potential of approximately 0.5 volts, and did not control the current. As a consequence, Harwig(132) required 1.5-4.5 hours for reaction time. In this thesis,a maximum current of 50 μA, combined with a maximum cell potential of 0.8 volt vs the saturated calomel electrode was found satisfactory.

Harwig et al.(132) found that they could not iodinate fibrinogen in phosphate buffer, but required a barbital buffer. Caro(125), Katz (123), Krohn(16), Malan(128), Rollag(127), and Sammon(124) reported the successful use of electrolyte containing phosphate. A number of workers used unbuffered saline, including Donabedian(126), Khalkhali(63), Pennisi(122), Rosa(120,23), and Teulings(121). In the experiments described in this thesis, a phosphate-sodium chloride buffer of ionic strength equal to 0.3 was found satisfactory.

The pH of the electrolyte used in radioiodinations was reported between 7.0 and 7.5. Teulings and Biggs(121) selected pH 8.0 on theoretical grounds, while Janonovic(130) used pH 10. Krohn(16) radioiodinated fibrinogen at pH 7.8. In the experiment dealing with pH, it was found that the yield decreased with increasing pH, particularly at pH 8.0. However, pH 7.0 was chosen based on the work of Hung(81) and Alexander(61,62).

The temperature used for electrolytic radioiodination was generally reported as room termperature. However, Sammon et al.(124) used 4°C; Rosa et al.(23) iodinated albumin at 8-10°C, and Krohn (16) labelled fibrinogen at 4°C. Pennisi(122) used a temperature of

5°C to improve the stability of the protein. In the experimental portion of this thesis, decreasing the temperature was found to decrease the yield.

Most reaction times reported were less than 30 minutes. A notable exception was Harwig(132) who reported reaction times of 1.5-4.5 hours, and Krohn(16) who labelled fibrinogen for 70-150 minutes. In the experimental portion of this thesis, it was found that 30 minutes was sufficient time to produce yields of 70 to 80%.

III. IN VITRO PROPERTIES OF ELECTROLYTICALLY RADIOIODINATED FIBRINOGEN

A. The Yield of Labelled Fibrinogen

Following electrolytic radioiodination of isolated human fibrinogen for 30 minutes at pH 7.0, the yield of labelled fibrinogen was determined by paper chromatography (Methods 1 and 4 fibrinogen), Sephadex chromatography (Method 2 fibrinogen), and trichloroacetic acid precipitation (Method 3 fibrinogen). The results are given in Table 19.

TABLE 19

The Yield of Labelled Fibrinogen by Electrolytic Radioiodination

Fibrinogen Isolated by:	Anolyte Volume	Fibrinogen Yield(%)	n	S.D.	S.E.M.
Method 1	1-2 ml	75.0	5	4.84	2.16
Method 2	0.3 ml	70.3	14	5.47	1.46
Method 3	0.6 ml	72.5	16	10.7	2.60
Method 4	1-3 ml	85.9	3	3.58	2.06
Method 5	2 ml	72.6			

B. The Clottability of Labelled Fibrinogen

The clottability of labelled fibrinogen was determined by the same method as that used for native fibrinogen. The clottability of labelled Connaught fibrinogen and human fibrinogen isolated by methods 1, 2, and 3 is presented in Table 20. The clottability of labelled canine fibrinogen isolated by methods 4 and 5 was not determined.

TABLE 20

The Clottability of Native and Labelled Fibrinogen

Fibrinogen	Form	% Clottability	n	S.D.	S.E.M.	Iodine/ Protein
Connaught	Native	88.7	6	1.2	0.48	
	Labelled	85.9	6	3.8	1.55	1:4
Method 1	Native	96.3	4	1.5	0.75	
	Labelled	94.7	12	2.2	0.64	1:2
Method 2	Native	93.6	11	2.1	0.64	
	Labelled	88.5	7	5.92	2.24	1:1
Method 3	Native	95.8	12	1.53	0.44	
	Labelled	95.6	12	4.14	1.20	1:1

It was first reported by McFarlane(20) that the number of iodine atoms substituted in each protein molecule has an effect on the metabolism of the labelled protein. He found that proteins substituted with 6 or more atoms of iodine (I/P = 6) were eliminated more rapidly than C-14 labelled proteins, but at 0.5 atom per molecule of protein, fibrinogen behaved the same way as C-14 labelled proteins.

However, Regoeczi(213) and Ly and Kierulf(211) found that there were no substitution dependent changes in the clottability of

iodine labelled fibrinogen. In addition, Harwig et al.(214) found that canine and human fibrinogens labelled up to I/P ratios of 4.5 demonstrated little change in biological clearance rate.

Student's t test was used to determine if the difference in clottability of the labelled fibrinogen was significantly different from the clottability of the native fibrinogen. In no instance was the difference significantly different at the 5% level using a one-tailed t-test. Thus, it appears that the labelling methods did not significantly affect the clottability of native fibrinogen.

C. Thrombin Clotting Time

The thrombin time has been suggested as a sensitive indicator of fibrinogen denaturation(211). This was measured on native and labelled Connaught Fibrinogen (iodine to protein ratio 1:4) and on native and labelled Method 3 fibrinogen (iodine to protein ratio 1:1); the results are presented in Table 21.

TABLE 21

The Thrombin Clotting Time of Native and Labelled Fibrinogen

Fibrinogen	Form	Thrombin Time (seconds)	n	S.D.	S.E.M.	Iodine/ Protein
Connaught	Native	29.3	8	3.4	1.20	1:4
	Labelled	27.8	8	3.5	1.25	
Method 3	Native	23.5	14	2.8	0.75	
	Labelled	21.6	13	2.0	0.56	1:1

The decrease in thrombin clotting time from the native to the labelled Connaught fibrinogen is not statistically significant at the

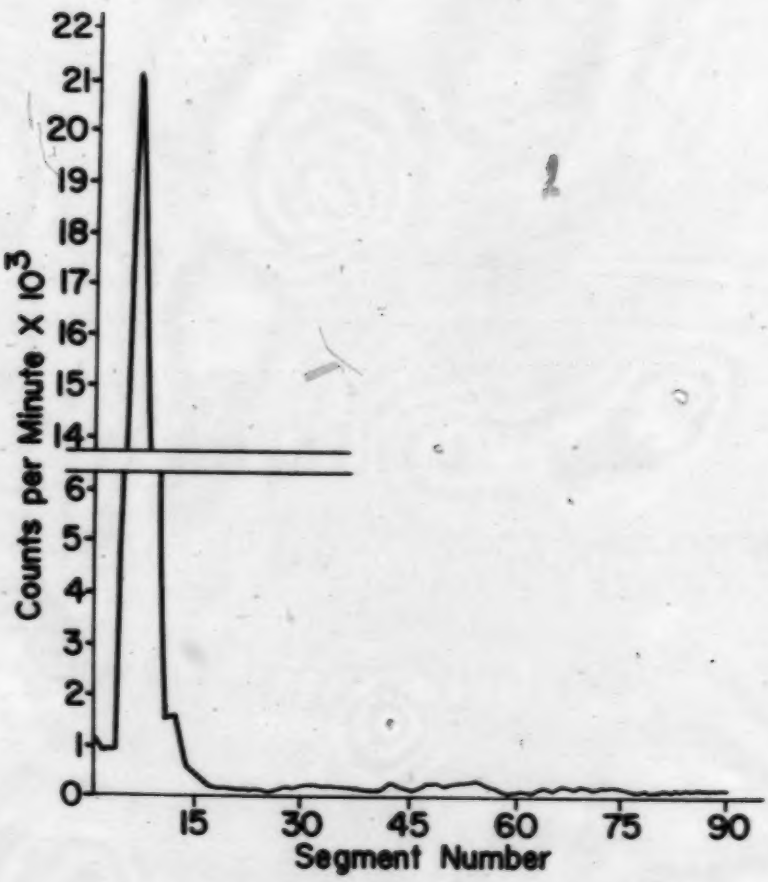
5% level as measured by the Student's t-test. However, it is significant for the difference between native and labelled Method 3 fibrinogen, and thus a true decrease in thrombin times exists for this highly clottable preparation. This decrease suggests that some alteration of the native state of the protein has occurred as a result of the iodination process. However, the decrease may also indicate an increased susceptibility of labelled fibrinogen to the enzymatic action of thrombin and which may, therefore, be preferentially taken up into forming thrombi.

D. Electrophoresis

Fibrinogen isolated by Method 1 was labelled by the electrolytic method and subjected to polyacrylamide gel electrophoresis (PAGE). A typical example of the distribution of activity in the gels is shown in Figure 15. This graph suggests that the iodinated fibrinogen is a single entity, and since the peak is symmetrical in appearance and no other areas of activity are observed, the labelled protein does not appear to be grossly denatured. Gels containing the labelled protein were stained as previously described and compared with gels containing unlabelled protein; they were identical in appearance.

Fibrinogen which had been isolated by Method 3 was subjected to electrophoresis before and after electrolytic radioiodination. Connaught fibrinogen and plasma were run as markers. The labelled and native proteins were identical in appearance, and consisted of a single discrete band.

Figure 15: Polyacrylamide Gel Electrophoresis of I-125 Fibrinogen



E. Radiochemical Stability of Labelled Fibrinogen

The rate of hydrolysis of I-125 from fibrinogen which was isolated by Method 2 was determined by removing 50 µl samples every 48 hours from the solution incubated at 37°C and chromatographing the aliquot on Sephadex G-10 + G-25. The procedure was performed on three different samples over a six day period. The average decrease in percent labelled fibrinogen was $2.2 \pm 0.59\%$ per day. It has been reported that the I-125 fibrinogen bond hydrolyzes rapidly in saline, and much more slowly in a solution of human serum albumin(16). It was suggested that electrolytic radioiodination of fibrinogen produces a large yield of sulfenyl iodide, by substitution of iodine for the hydrogen of the sulfhydryl group on cysteine(132). Diluting the anolyte reaction mixture with distilled water produced an ionic strength equal to saline; dilution of anolyte with cysteine solution provided a system for sulfenyl iodide in the protein to exchange with the free sulfhydryl groups of the cysteine. Dilution with human serum albumin and serum provided a protein solution which may provide a protective medium for the fibrinogen.

The rate of hydrolysis of I-125 from Connaught fibrinogen labelled at an initial ratio of 1 iodide atom per 4 protein molecules was measured daily for six days. The reaction mixture was mixed 1:1 with each of: distilled water, 1×10^{-3} M cysteine, 12.5% albumin, and human serum. The samples were prepared in duplicate. The rate of hydrolysis of I-125 from labelled Connaught fibrinogen in pH 7.0 buffer (phosphate - NaCl, $\mu = 0.3$) was determined in triplicate.

The results of these determinations are presented in Table 22.

The hydrolysis rate of electrolytically labelled human fibrinogen is between 2 and 4% per day. This is considerably less than the 27% per day previously reported for this method(16).

F. Sepharose Chromatography

The molecular weight profiles of native and labelled fibrinogen were determined by chromatographing protein solutions on Sepharose 2B and 6B. Two peaks were generally observed; the large peak was centered at approximately 90 ml of eluate, and a smaller peak was observed at half this elution volume at 45 ml. This small peak was considered to contain aggregated material. Metzger et al.(131) found that when aggregated fibrinogen was injected into laboratory animals, it was rapidly cleared from the circulation and accumulated in the liver and spleen.

Some workers(16) had found that some aggregated material was too large to enter the gel; therefore, the Sepharose columns were back-washed with a volume of eluate equal to the elution volume of iodide (100 ml). The area under the elution profile curve was determined by a gravimetric method and the area of the aggregate peak was expressed as a percent of the total area. The elution profiles of labelled and unlabelled fibrinogen are shown in Figure 16, and the percent of aggregation in native and labelled fibrinogen obtained from Connaught Laboratories and by isolation from plasma by Method 1 are given in Table 23.

As measured by area, the Connaught fibrinogen contained 0.17%

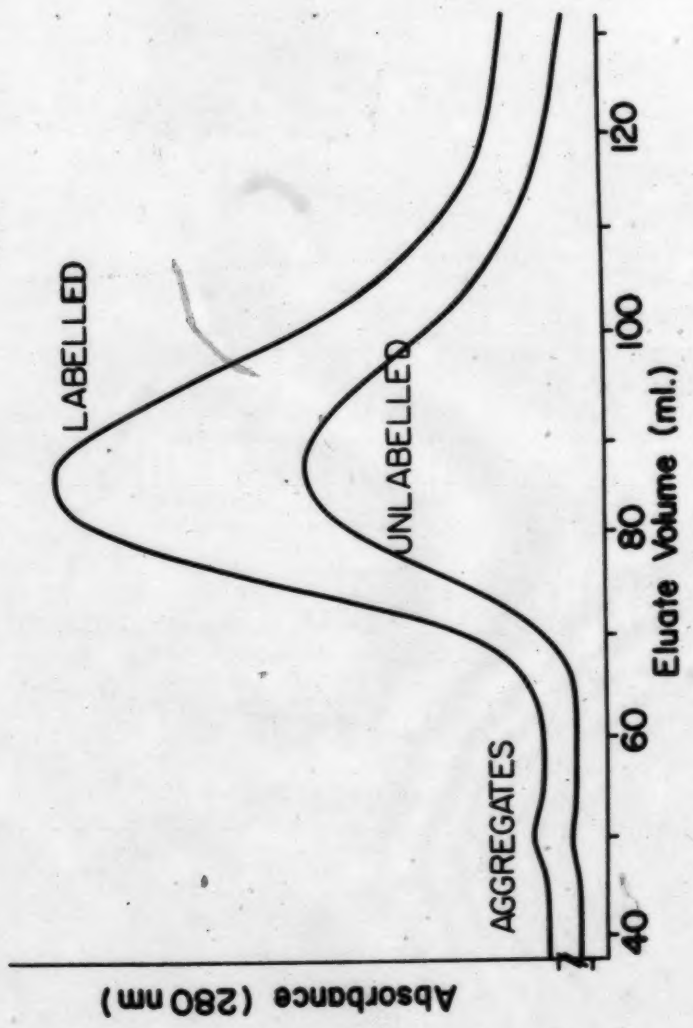


Figure 16: Fractionation of Labelled and Unlabelled Fibrinogen on Sepharose 6B

TABLE 22

The Rate of Hydrolysis of Electrolytically Radioiodinated Human Fibrinogen

Time (days)	Percent of I-125 Hydrolyzed at 37°C					
	Method II Fibrinogen	Connaught Fibrinogen				
		pH 7.0 Buffer	Diluted pH 7 Buffer	Cysteine	Albumin	Serum
1		0.0	0.33	4.7	0.0	0.09
2	3.1	2.4	5.5	2.8	3.2	3.0
3		1.5	3.3	3.6	5.4	5.5
4	5.4	5.1	0.32	2.1	3.6	2.0
5		4.2	3.1		0.74	4.1
6	4.7	3.2	3.3		0.0	0.0
7		6.5				
Mean % per day	2.2	3.3	2.64	3.30	2.15	2.44
S.D.	0.59	2.2	1.99	1.11	2.22	2.19
S.E.M.	0.34	0.83	0.81	0.55	0.91	0.89

TABLE 23

Sepharose Chromatgoraphy Determination of Aggregation in Native and Labelled Fibrinogen

Fibrinogen	Area of Void Peak			Radioactivity in the Void Peak			Radioactivity in the Back Wash		
	%	S.D.	S.E.M.	%	S.D.	S.E.M.	%	S.D.	S.E.M.
Connaught									
Native	9.17	1.13	0.57(n=4)						
Labelled	9.14	0.64	0.32(n=4)	8.72	0.71	0.32(n=5)	1.04	0.24	0.12(n=5)
Method 1									
Native	3.23	1.37	0.56(n=6)						
Labelled	4.19	2.11	0.75(n=8)	0.512	0.26	0.11(n=6)	0.91	0.278	0.11(n=6)

more aggregates after electrolytic radioiodination, while Method 1 fibrinogen contained 0.96% more aggregated protein after labelling Method 1 fibrinogen contained 1.43% of aggregated material as measured by assaying the radioactivity in the void peak and in the backwash. This is considerably less than the 47% aggregated content of electrolytically labelled fibrinogen reported in the literature(11).

In summary, the yield of labelled fibrinogen varied between 70.3% and 85.9%. The decrease in clottability after labelling was not statistically significant at the 5% level. However, the decrease in thrombin clotting time was significant at the 5% level for the highly clottable preparation. No degradation was detected by polyacrylamide gel electrophoresis. The rate of hydrolysis of I-125 from fibrinogen in various media ranged between 2% and 4% per day in buffer solutions and in protein containing media. The increase in aggregation produced by electrolytic radioiodination was not statistically significant.

Krohn et al(16) reported that 15.6% to 17% of electrolytically labelled fibrinogen remained on top of a Sepharose 4B column. These workers found that the yield of fibrinogen was 50% and the clottability decreased from 97% to 93% after labelling. The rate of hydrolysis was 1.5% per day in plasma and 1.24% in albumin. In the experimental portion of this thesis, the hydrolysis rate of labelled fibrinogen was found to be 2.15% in albumin and 2.44% per day in serum. In saline, Krohn(16) found that the hydrolysis rate was 27% in 1 day and 51% in three days. In contrast, the experiments described in this thesis suggested that the hydrolysis rate in buffer of ionic strength

equal to 0.15 (the same as saline) was 0.33% in one day and 9.1% in three days. Krohn and Welch(119) subsequently reported a yield of labelled fibrinogen by the electrolytic method of 30-80%, and an isotopic clottability of 79% with a 27% per day hydrolysis rate. The amount of aggregation by gel permeation chromatography was about 50%(119).

IV. THE IN VIVO BEHAVIOUR OF ELECTROLYTICALLY RADIOIODINATED FIBRINOGEN

A. The Clearance of Canine Fibrinogen in Dogs

The clearance rate of canine fibrinogen isolated by Method 4 was measured in two dogs. The fibrinogen was isolated from an average volume of 31.5 ml of citrated canine plasma. The fibrinogen was electrolytically radioiodinated for 30 minutes; the anolyte volume was between 1 to 2 ml and contained an average 5.8×10^{-5} M protein and 2.5×10^{-5} M KI, resulting in an average iodine to protein ratio of 1 to 2. Three hundred microcuries of labelled fibrinogen was injected into each dog, after the free iodine was removed by chromatographing the anolyte on a 0.9 cm x 30 cm column of G-25 Sephadex equilibrated with pH 7.4 isotonic phosphate buffer.

Since it was found that Method 1 of isolating human fibrinogen could not be applied clinically, canine fibrinogen isolated by Method 4 (the same as Method 1, except canine plasma was used) was not tested in more dogs. Instead, canine fibrinogen isolated by Method 5 was further tested; Method 5 is similar to Method 3 of

isolating human fibrinogen and this latter method was sufficiently simple and rapid that it could be adapted to clinical use.

Method 5 canine fibrinogen was tested in four dogs. It was isolated from 800 ml of canine plasma, and was electrolytically radioiodinated for 30 minutes. The 2.2 ml anolyte contained 4×10^{-5} M fibrinogen and 2.7×10^{-5} M KI, and an iodide to protein ratio of 1 to 1.5. Three hundred microcuries was injected into each dog.

Blood samples were collected as described previously and the radioactivity in 0.5 ml plasma samples was determined using a Searle 1195 Gamma Spectrometer. The log of counts per minute versus time in hours were analyzed by a stripping method on a Digital PDP-11 computer (Digital Corporation, Maynard, Mass.) interfaced to a Northern Scientific NS-636 Multi-Channel Analyzer and a Dicom Tape Deck.

A single exponential was fitted to the data for the long lived component using a weighted least squares method. The contribution of this long-lived component was subtracted from the earlier data and the second longest lived component was then determined in a similar manner. The weighting factor in the program took into account the diminishing precision of the data as components were removed, as well as the distortion required to accommodate an exponential rather than a linear fit. The precision of the fitting was indicated in terms of the standard deviations of both the size of each component and its corresponding elimination constant.

The results of these stripping procedures are given in Table 24. Fibrinogen isolated by Method 4 was eliminated in two

TABLE 24

Canine Fibrinogen Plasma Clearance Rates

Method 4

Component	Elimination Constant 'a'	S.E.M.	Intercept 'C'	S.E.M.	Half-Life (Hours)	S.E.M.	n
1	9.01×10^{-3}	5.13×10^{-4}	0.8347	0.059	77.2	4.39	2
2	0.1551	-	0.1452	-	4.8	-	1

Method 5

Component	Elimination Constant 'a'	S.E.M.	Intercept 'C'	S.E.M.	Half-Life (Hours)	S.E.M.	n
1	9.32×10^{-3}	4.36×10^{-4}	0.5189	0.062	74.9	3.42	4
2	1.97×10^{-2}	2.21×10^{-3}	0.3716	0.063	36.6	4.60	4
3	0.1029	2.21×10^{-3}	0.1153	0.0498	7.3	1.29	3

exponential phases, the first being a mixing and equilibrium phase ($t_{\frac{1}{2}}$ = 4.8 hr.) and the second represents the catabolism of fibrinogen ($t_{\frac{1}{2}}$ = 77.2 hr.). The fibrinogen isolated by Method 5 was eliminated in three exponential phases, the latter two exponentials representing the catabolism of fibrinogen. (A clearance curve of plasma radioactivity of labelled Method 4 fibrinogen is shown in Figure 17, and a clearance curve of plasma radioactivity of labelled Method 5 fibrinogen is shown in Figure 18.) The problem of the double exponential elimination of fibrinogen has been reviewed by Regoeczi (212) who believed that the phenomenon is due to old, out-dated preparations or to poor storage of the protein. The mixing and equilibrium phase had a half-life of 7.3 hours. The second exponential phase had a half-life of 36.6 hours, and the third exponential had a half-life of 74.9 hours.

Metzger et al.(131) attributed the second exponential to the release of iodide from the fibrinogen in vivo, and found that the amount of unbound iodide ranged from 20% to 80% for Chloramine T labelled fibrinogen and 20% to 57% for ICl labelled fibrinogen(131). The average unbound iodide found in these studies was 1.4% as measured by TCA precipitation. This amount was deemed small enough that no further correction of the data was necessary for the content of free iodide.

The double half-life encountered in these experiments may be due to the presence of activated clotting factors in the blood of the donor animal, which had under gone extensive surgery before the blood was removed.

The fractional catabolic rate (FCR) is a measure of the rate

Figure 17: Plasma Clearance Curve of I-125 Fibrinogen and the Two Components Derived by a Computorized Stripping Technique

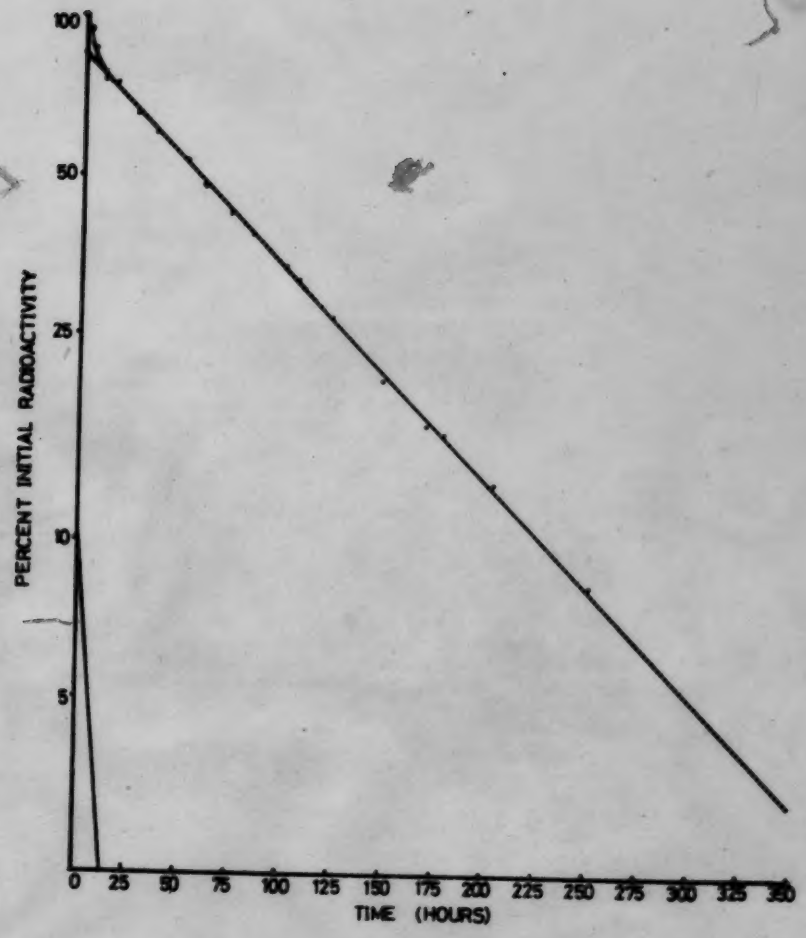
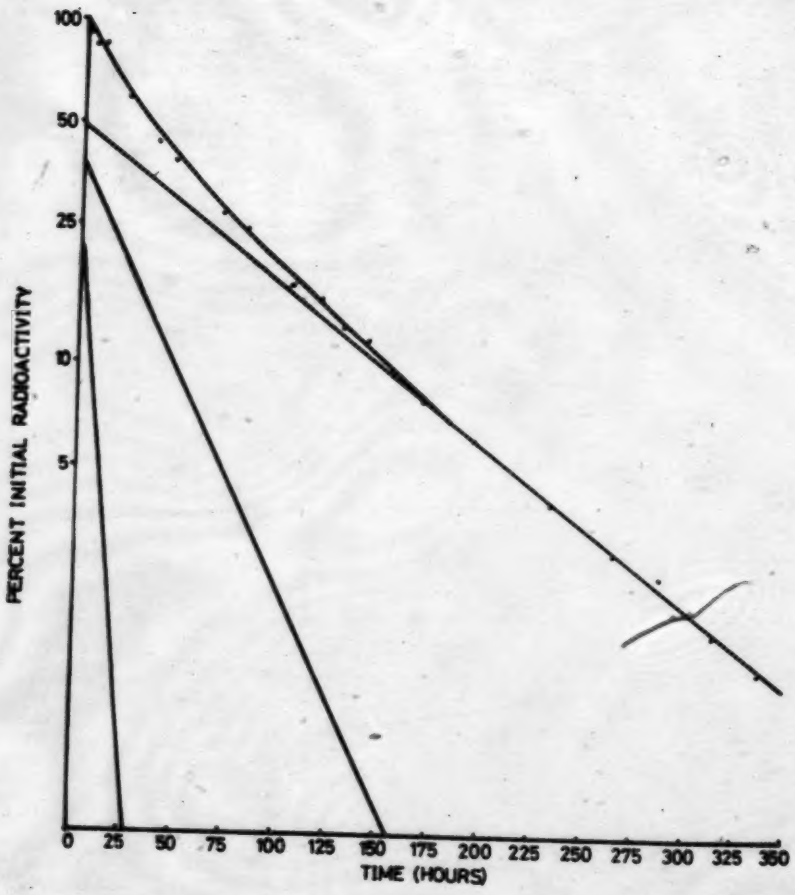


Figure 18: Plasma Clearance Curve of I-125 Fibrinogen and the Three Components Derived by a Computorized Stripping Technique



at which a protein is metabolized. It is stated as a fraction of the injected dose eliminated per unit time. In metabolic studies, it is assumed that the FCR is equivalent to the fraction of the body pool of the protein eliminated per unit time. The smaller the FCR, the slower the rate of clearance of the protein from the plasma. Depending on the model used to study the metabolic parameters of the protein, the FCR may be a function of two or more half-lives or elimination constants.

Since thrombosis may occur in the post-operative patient several days or weeks after surgery, it is advantageous to use fibrinogen I-125 with the longest possible biological half-life or lowest FCR, thereby providing the longest possible surveillance for detection of thrombosis.

The fractional catabolic rates for these two sets of fibrinogen data can be calculated from the following equations:

1. Method 4 Fibrinogen: Campbell's Two Compartmental Model(271):

$$k_m = \frac{1}{\frac{C_1}{a_1} + \frac{C_2}{a_2}} = \frac{1}{\frac{0.8347}{9.01 \times 10^{-3}} + \frac{0.1452}{0.1551}} = 1.07 \times 10^{-2} \ hr^{-1}$$

k_m - fractional catabolic rate

C_1 - y intercept at time 0 for the first component

a_1 - elimination rate constant of the first component

C_2 - y intercept at time 0 for the second component

a_2 - elimination rate component of the second component

C_3 - y intercept at time 0 for the third component

a_3 - elimination rate constant of the third component

2. Method 5 Fibrinogen: Matthew's Three-Compartment Open Parallel Model:

$$k_m = \frac{1}{\frac{C_1}{a_1} + \frac{C_2}{a_2} + \frac{C_3}{a_3}} = \frac{1}{\frac{0.5189}{9.32 \times 10^{-3}} + \frac{0.37.6}{1.97 \times 10^{-2}} + \frac{0.1153}{0.1029}}$$

$$= 1.32 \times 10^{-2} \text{ hr}^{-1}$$

The fractional catabolic rate of Method 4 fibrinogen is 18.9% lower than that of Method 5. Since it is known that the second exponential in the double half-life fibrinogen is an artifact, the intercepts of Method 4 can be modified to eliminate the second exponential:

$C_1' = C_1/(C_1 + C_3)$ and $C_2' = C_3/(C_1 + C_3)$

$C_1' = 0.5189/(0.5189 + 0.1153)$

$= 0.8182$

$C_2' = 0.1153/(0.5189 + 0.1553)$

$= 0.1818$

The fractional catabolic rate for Method 5 fibrinogen can be recalculated using Campbell's Two Compartment Model:

$$k_m = \frac{1}{\frac{C_1'}{a_1} + \frac{C_2'}{a_1}} = \frac{1}{\frac{0.8182}{9.32 \times 10^{-3}} + \frac{0.1818}{0.1029}} = 1.12 \times 10^{-2}$$

The fractional catabolic rate of Method 4 fibrinogen is now 4.2% lower than the recalculated FCR for Method 5.

A better estimate of the values C_1 and C_2 can be obtained from the average of the values from Method 4 results and the recalculated values of Method 5:

$C_1 = 0.8265$ and $C_2 = 1 - C_1 = 0.1736$

The fractional catabolic rate can then be recalculated to give the

best estimate of k_m:

$$k_m = \frac{1}{\frac{C_1}{a_1} + \frac{C_2}{a_2}} = 1.093 \times 10^{-2}\ hr^{-1} = 0.262\ d^{-1}$$

Using the same values of C_1, a_1, C_2 and a_2, the rates of transfer of protein to and from the extravascular compartment can be determined respectively:

$$K_{1,2} = C_1C_2(a_2 - a_1)^2/(C_1a_2 + C_2a_1)$$

$$= 1.904 \times 10^{-2}\ hr^{-1}$$

$$= 0.457\ day^{-1}$$

In this case, 45.7% of the intravascular pool is transferred to the extravascular pool per day.

$$k_{2,1} = C_1a_2 + C_2a_1 = 0.1082\ hr^{-1} = 2.60\ d^{-1}$$

Fibrinogen in the extravascular pool is returned to the intravascular pool at the rate of 260% per day.

Dugan et al.(206)reported that fibrinogen labelled by the chloramine-T method was metabolized by dogs in two exponential components, half of the injected dose being eliminated in 2.2 hours, and the other half in 103.2 hours. Using Campbell's two compartmental model, a fractional catabolic rate of $1.32 \times 10^{-2}\ hr^{-1}$ can be calculated. This is 1.2 times faster than the fractional catabolic rate determined in the experimental portion of this thesis for fibrinogen eliminated in two exponential components, namely $1.07 \times 10^{-2}\ hr^{-1}$. Similarly, Tytgat et al.(201)reported a fractional catabolic rate of 1.63×10^{-2} for iodine monochloride labelled fibrinogen which was eliminated in two exponential components in

dogs. This 1.5 times greater than the fractional catabolic rate calculated for the two compartment model of fibrinogen metabolism in this thesis.

Metzger et al.(131) studied the elimination rate of fibrinogen labelled by the iodine monochloride method, and found that it was superior to lactoperoxidase iodination, and very much superior to chloramine-T and electrolytic labelling. The rate of elimination of iodine monochloride fibrinogen was measured in dogs and these workers found that it was eliminated in three exponential components. Using Matthew's three-compartmental model, a fractional catabolic rate can be calculated for their data, and this was found to be 4.35×10^{-2} hr^{-1}. This rate is 3.2 times faster than the elimination rate determined in the experimental portion of this thesis for fibrinogen eliminated in three exponential components, which was found to be 1.32×10^{-2} hr^{-1}.

These results suggest that the electrolytic method of radioiodination of fibrinogen produces a product which is at least as satisfactory (if not better) as the iodine monochloride and chloramine-T preparations with regard to rate of elimination from the plasma of dogs.

B. Clearance Rates of Labelled Human Fibrinogen

Rabbits

Analysis of curves for clearance of electrolytically labelled human fibrinogen in rabbits indicated that 40% of the injected dose was cleared with a half-life 54.2 hours while the remaining 60% was

cleared with a half-life of 10 hours. The fractional catabolic rates of electrolytically radioiodinated fibrinogen and of several commercial preparations were calculated from the following formula (279):

$$k_m = \frac{\ln 2}{(C_1 \times t_{\frac{1}{2}_1}) + (C_2 \times t_{\frac{1}{2}})}$$

The results are presented in Table .

TABLE 25

Human Fibrinogen Plasma Clearance Rates in Rabbits

Method of Labelling Human Fibrinogen	Fractional Catabolic Rate (pool fraction/hr)
Iodine Monochloride	0.044
Iodine Monochloride	0.041
Chloramine-T	0.043
Electrolytic	0.025

Thus, the FCR of electrolytically radioiodinated human fibrinogen in rabbits is 42.5% lower than the average FCR of commercial preparations.

The average amount of free iodide in rabbit plasma as measured by TCA precipitation was 0.6% at 15 minutes and 1.2% at four hours. Electrophoresis of the product on Gelman polyacetate strips, using Gelman high resolution barbital buffer pH 8.8, indicated that 75.3% migrated similarly to pure fibrinogen. The remaining 24.7% failed to migrate from the origin; the commercial products showed from 30-55% of the activity at the origin, with only 45-70% running as monomeric fibrinogen.

Electrophoresis of rabbit plasma samples at fifteen minutes showed that 96.8% of the total activity in the plasma migrated with the fibrinogen peak and 2.8% remained at the origin. Analysis of plasma samples obtained after four hours indicated that 92.6% of the activity ran with the fibrinogen peak, while 5.8% remained at the origin. Positions of the proteins were determined by running non-labelled albumin and fibrinogen (Connaught Laboratories) and staining the electrophoretograms with Ponceau S.

V. IMAGING OF SURGICALLY INDUCED THROMBI IN LEG VEINS OF DOGS

The results of various imaging procedures, which were carried out for a maximum of 5 days after induction of the thrombus, are given in Table 26. These results suggest that the fibrinogen preparation can be used to detect thrombi shortly after they are induced. Induction by electrocauterization (Trials 3 to 6) appears to be a superior method of induction since the venograms indicated that cauterization of the intima produced only partial occlusion by the thrombus, which lyzed in three days. The use of a thrombin soaked thread (Trials 1 and 2) produced total occlusion of the vein and swelling of the extremity. Figures 19 and 20 show multi-wire proportional chamber scans of thrombi produced by the thrombin soaked thread procedure and the electrocautery method respectively.

The multi-wire proportional chamber is particularly suited for the detection of deep venous thrombosis by means of the I-125 fibrinogen uptake test. The device is most sensitive for low energy gamma radiation and less sensitive as the energy of the radiation

TABLE 26

Canine Thrombosis Studies

Trial	Site of Thrombosis	Scan Method	Time(Days) 1	2	3	4	5
1	Right Rear Calf	MWPC*	++	o	o	o	++
		Rectilinear	-	o	o	+	o
		Venogram	+	o	o	o	o
2	Right Rear Calf	MWPC*	o	o	o	o	o
	Sham operated	Rectilinear	++	+	-	o	o
	Left rear Calf	Venogram	o	o	o	o	o
3	Right Rear Thigh	MWPC*	o	o	o	o	o
	Left Rear Thigh	Rectilinear	-	-	-	o	o
		Venogram	o	o	o	o	o
4	Right Rear Thigh	MWPC*	o	o	o	o	o
		Rectilinear	-	-	-	o	o
		Venogram	o	o	o	o	o
5	Right Rear Thigh	MWPC*	+	+	-	o	o
		Rectilinear	o	o	o	o	o
		Venogram	+	o	o	o	o
6	Left Rear Thigh	MWPC*	o	o	o	-	o
		Rectilinear	++	+	+	-	o
		Venogram	o	o	-	o	o

Symbols

++ good resolution
+ fair resolution
± poor resolution
- absent
o procedure not performed
* Multi-wire Proportional Chamber

Figure 19: Multi-Wire Proportional Chamber Image of a Superficial Thrombus in the Rear Leg of a Dog Injected with I-125 Fibrinogen

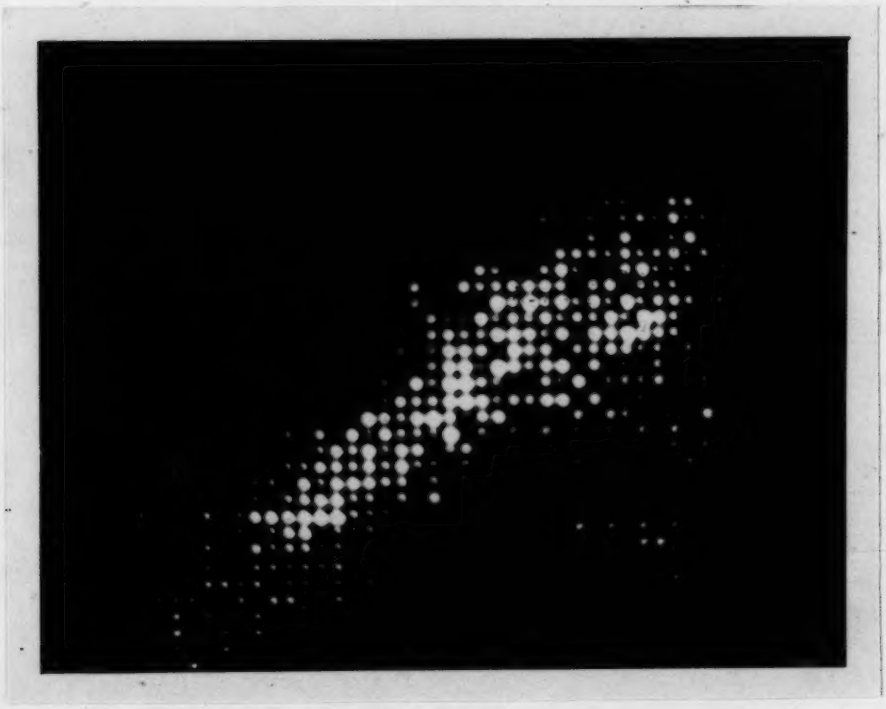


Figure 20: Multi-wire Proportional Chamber Image of a Thrombus in the Deep Veinous System in the Rear Leg of a Dog Injected with I-125 Fibrinogen

increases. The chamber can be built to any dimensions, such as the length and width of the calf. Thus, the entire calf (or the entire thigh) could be imaged at one time. The chamber and its associated electronics are relatively small and light weight, and could be brought to the patient's bedside. The combination of a good fibrinogen preparation and the multi-wire proportional chamber appear to be a powerful tool for the investigation of the natural history of thrombosis.

SUMMARY AND CONCLUSIONS

1. Various parameters for electrolytic radioiodination were investigated. Optimum yields of labelled fibrinogen could be obtained when the anolyte volume was the smallest possible for a particular cell design. A satisfactory electrolyte was found to be pH 7.0 phosphate-sodium chloride buffer of ionic strength equal to 0.3. The yield of labelled fibrinogen was found to be independent of catholyte volume. The procedure could be best carried out at room temperature, rather than at lower temperatures. The optimum voltage was found to be 0.8 volt versus the saturated calomel electrode; the optimum current was found to vary with the anolyte volume, being 50 μA for 1.0 ml and 10 μA for 0.3 ml. A satisfactory technique of electrolysis was to apply the optimum current to the cell until the voltage reached 0.8 volt vs SCE, and then allow the current to decrease exponentially, while the voltage was maintained at 0.8 volt. The optimum iodide and protein concentrations was found to be 3×10^{-5} M. Thirty minutes was found to be sufficient time to produce a yield between 70% and 80%.
2. A rapid precipitation technique was developed for isolation of human fibrinogen. An average quantity of 5.5 mg of fibrinogen was isolated from an average volume of 5.2 ml of plasma. The preparation was stabilized by the use of an adsorbent to remove the prothrombin complex and by the use of epsilon-amino caproic acid to inhibit plasminogen.
3. Isolation of fibrinogen by means of a large column of Sephadex G-200 was unsatisfactory and impractical with regard to its

speed and sterility.

4. The yield of iodination reaction was determined by means of a small column consisting of 75% Sephadex G-25 and 25% G-10. This procedure was found to be more accurate and less time consuming than more conventional methods.

5. Pre-packed, disposable Sephadex G-25 columns were found to be satisfactory for removing unbound iodide from the iodinated fibrinogen.

6. The decrease in clottability after labelling by the electrolytic method ranged from 0.2% for a good fibrinogen preparation to 5.1% for an unsatisfactory preparation. There was a slight decrease in thrombin clotting time, which suggested that iodination might induce a change in the fibrinogen molecules which rendered them more susceptible to the action of thrombin.

7. No change in chromatographic behaviour of labelled fibrinogen compared to native fibrinogen was observed by polyacrylamide gel electrophoresis or agarose gel electrophoresis.

8. The rate of hydrolysis of radioiodine from fibrinogen ranged from 3.3% per day in cysteine solution to 2.15% per day in albumin, measured at 37°C.

9. It was found that electrolytic radioiodination did produce aggregate formation in the fibrinogen preparation, but this was less than 1%.

10. The clearance rate of electrolytically radioiodinated canine fibrinogen was studied in dogs. It was found that one type of preparation was cleared from plasma in two exponential components, the slow component with a half-life of 77.2 hours

and a rapid component with a half-life of 4.8 hours. The second preparation was found to be cleared in three exponential components, the longest with a half-life of 74.9 hours, the intermediate component with a half-life of 36.6 hours, and the shortest component with a half-life of 7.3 hours.

11. Since it is known that the intermediate half-life is not always demonstrable, this component was eliminated mathematically, and a corrected fractional catabolic rate was determined. The best estimate of canine fibrinogen catabolic rate was considered to be 0.262 of the pool per day.

12. The fractional catabolic rate of electrolytically labelled human fibrinogen in rabbits was determined to be 0.025 of the pool per hour. This was found to be 42.5% lower than the fractional catabolic rate for iodine monchloride and chloramine-T labelled human fibrinogen preparations.

13. Human fibrinogen was found to be cleared from the blood of rabbits in two exponential phases. The slow component was cleared with a half-life of 54.2 hours, and the rapid component was cleared with half-life of 10 hours.

14. The results of several qualitative studies of the imaging of surgically induced thrombi in dogs suggest that electrolytically radioiodinated fibrinogen selectively accumulated in the forming thrombi to the extent that the thrombus could be imaged by multi-wire proportional chamber and by a rectilinear scanner.

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APPENDIX I

1.	a	elimination constant
2.	ala	alanine
3.	arg	arginine
4.	C	zero time intercept
5.	DIH	diiodohistidine
6.	DIT	diiodotyrosine
7.	DVT	deep venous thrombosis
8.	EACA	epsilon aminocaproic acid
9.	ev	extra-vascular compartment
10.	FCR	fractional catabolic rate
11.	HCG	human chorionic gonadotrophin
12.	HGH	human growth hormone
13.	I/P	iodine to protein ratio
14.	IV	intra-vascular compartment
15.	km	fractional catabolic rate
16.	LP	lactoperoxidase
17.	μA	microampere
18.	μl	microliter
19.	MIH	monoiodohistidine
20.	MIT	monoiodotyrosine
21.	N-DSK	N-terminal disulfide knot
22.	pro	proline
23.	Pt vs Pt	platinum vs platinum cell
24.	Pt vs SCE	platinum vs saturated calomel electrode cell
25.	SCE	saturated calomel electrode

26.	SD	standard deviation
27.	SEM	standard error of the mean
28.	T-3	tri-iodothyronine
29.	T-4	thyroxine
30.	TEAE Cellulose	tetraethylaminoethyl cellulose - cation exchange resin
31.	TP	thyroid peroxidase
32.	Tris	tris (hydroxymethyl) amino methane
33.	TSH	thyroid stimulating hormone
34.	Z	N-benzyloxycarbonyl